DEVELOPMENT OF GEL FORMULATIONS OF SYNTHESIZED METHYL SALICYLATE AND ITS EFFECT ON ENZYME INHIBITION ASSAY OF COX-II

Original Research Article

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ABSTRACT: Methyl Salicylate (Wintergreen oil) is a topical analgesic and anti-inflammatory agent. A topical nonsteroidal anti-inflammatory drug (NSAID). Methyl salicylate lactoside is a COX inhibitor. Selective COX-2 inhibitors increase the risk of myocardial infarction and stroke. The actual aim of this research was to synthesize the methyl salicylate and development of a gel formulation with MS and to get a confirmation whether MS having any selective COX II inhibition effect or not. Yield of methyl salicylate was 81% with R.F. value 0.67. The IR spectra of MS was compared with that of the standard peak available in earlier reported articles and identified as MS. But these maximum % inhibition values were similar at the concentration of 250mcg/ml and it was approximately 94%. Although the values of % inhibition at the concentration of 500mcg/ml was little bit lower than the values at 250 and 1000, but it was greater than the PC. From this table it can be concluded that 250mcg/ml will be best among all the COX II enzyme inhibition assay of methyl salicylate. Cox II enzyme inhibition assay of MS Gel is tabulated in Table 5, in which it was mentioned the Log (inhibitor) vs. normalized response and variable slope with 95% Confidence Intervals of IC50 values and it was in the range of 0.1510 to 1.228, but best fit value of IC50 was 0.4307. Goodness of Fit value was statistically evaluated and also calculated in which R2 value was 0.9626, which itself prove that it was most linear and within the acceptable limit. Finally, it can be concluded that MS gel having sufficient COX II enzyme inhibition properties and which may be beneficial as compared to the others having similar to them.

KEYWORDS: Methyl salicylate; COX II enzyme inhibition; FTIR study, IC50.

INTRODUCTION

Methyl Salicylate (Wintergreen oil) is a topical analgesic and anti-inflammatory agent. A topical nonsteroidal anti-inflammatory drug (NSAID). Methyl salicylate lactoside is a COX inhibitor [1]. COX-2 inhibitors are a type of NSAID. They treat the pain and inflammation of many types of arthritis and other types of short-term pain. COX-2 inhibitors are as effective as traditional NSAIDs but cause less stomach and intestinal problems. Celecoxib is the only COX-2 inhibitor available in the U.S. COX-2 inhibitors may increase the risks of heart attacks and stroke and stomach and intestinal problems [2]. Selective COX-2 inhibitors currently used in the clinic are the sulphonamides celecoxib and valdecoxib (parecoxib is a prodrug of valdecoxib), as well as the methyl sulphones rofecoxib and etoricoxib. Furthermore, the phenylacetic acid derivative lumiracoxib has gained permission recently to be marketed in Europe [3]. NSAIDs inhibit COX, thereby inhibiting prostaglandin production. Two COX enzymes are known to be involved in prostaglandin synthesis, COX-1 and COX-2. COX-1 generates prostaglandins that are involved in the protection of gastrointestinal mucosa, while COX-2 generates prostaglandins that mediate inflammation and pain in sites throughout the body. Selective COX-2 inhibitors may therefore relieve the pain of inflammation without deleterious effects on gastrointestinal mucosa [4].

Aspirin, one of the oldest and most common anti-inflammatory agents, has recently been shown to reduce cancer risks. Selective NSAIDs (also called COX-2 inhibitors) are as effective in relieving pain and inflammation as nonselective NSAIDs and are less likely to cause gastrointestinal injury. Celecoxib (Celebrex) is a selective NSAID that is available in the United States. Other selective NSAIDs that can be found elsewhere in the world include etoricoxib (Arcoxia) and lumiracoxib (Prexige). Selective NSAIDs are sometimes recommended for people who have had a peptic ulcer, gastrointestinal bleeding, or gastrointestinal upset when taking nonselective NSAIDs. Selective NSAIDs have less potential to cause ulcers or gastrointestinal bleeding [5,6].

Selective COX-2 inhibitors increase the risk of myocardial infarction and stroke. This has been attributed to their ability to inhibit endothelial COX-2 derived prostacyclin (PGI2) but not platelet COX-1 derived thromboxane A2 (TXA2). On the other hand, aspirin blocks both COX-1 and COX-2 enzymes without decreasing PGI2 but blocks TXA2 synthesis that explains its beneficial action in the prevention of coronary heart disease (CHD). The inhibitory action of aspirin on COX-1 and COX-2 enzymes enhances the tissue

concentrations of dihomo-gamma-linolenic acid (DGLA), arachidonic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). [7]

By specifically only blocking COX-2 enzymes, COX-2 inhibitors relieve inflammation and pain with less adverse gastrointestinal effects than NSAIDs that inhibit both COX-1 and COX-2 enzymes. However, they are not devoid of gastrointestinal effects entirely, and their use (like all NSAIDs) has been associated with a higher risk of stroke and heart attack.[7-9] COX inhibitors divide into non-selective nonsteroidal anti-inflammatory drugs (NSAIDs), COX-2 selective nonsteroidal anti-inflammatory drugs (c2s NSAIDs), and aspirin. NSAIDs include ibuprofen, naproxen, ketorolac, and indomethacin. C2s NSAIDs only include celecoxib. Meloxicam and diclofenac are cox-inhibitors that are not categorized. [8-10]

COX-1 catalyzes formation of cytoprotective prostaglandins in thrombocytes, vascular endothelium, stomach mucosa, kidneys, pancreas, Langerhans islets, seminal vesicles, and brain. Induction of COX-2 by various growth factors, proinflammatory agents, endotoxins, mitogens, and tumor agents indicates that this isoform may have a role in induction of pathological processes, such as inflammation [11-12]. The enzyme exists in at least 2 isoforms. COX-1 generates prostaglandins with physiological functions, COX-2 is induced by inflammation and its physiologic functions are unclear at present. Conventional NSAIDs, like diclofenac, ibuprofen, and naproxen, are non-selective COX inhibitors, blocking the production of both physiologic and inflammatory prostaglandins [13].

Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used to treat pain associated with a variety of medical conditions. Nonselective NSAIDs reversibly inhibit the enzyme cyclooxygenase (COX) in both of its isoforms, COX-1 and COX-2. An increased risk of cardiovascular events has been associated with the use of NSAIDs, especially of COX-2 selective NSAIDs. Current evidence suggests that naproxen, a nonselective NSAID, is associated with the lowest risk of cardiovascular events. Therefore, naproxen is the NSAID of choice in patients with high cardiovascular risk [14-15].

The actual aim of this research was to synthesize the methyl salicylate and development of a gel formulation with MS and to get a confirmation whether MS having any selective COX II inhibition effect or not.

MATERIALS AND METHODS

MATERIALS

Salicylic acid was procured from CDH lab and others all the chemicals and reagents were laboratory grade.

METHODS

SYNTHESIS OF METHYL SALICYLATE

0.65gm of salicylic acid and 10ml methanol with a magnetic stir bar were placed in a round bottom flask. Stirred the mixture until the salicylic acid dissolved with the help of magnetic stirrer. Placed the conical flask on a stirring hotplate and while stirring slowly and in small portions 0.75 ml of concentrated sulfuric acid was added to the salicylic acid and methanol solution. Attached the round flask to a water-cooled condenser and cap the condenser with a drying tube that had been loosely packed with $CaCl_2$. The solution was gently boiled for 75 minutes maintaining the temperature at approximately 100°C. Shaked the resulting suspension. Allowed the layers to separate and transferred the organic layer which contains methyl salicylate to another container. Mixed the solutions together and allowed the solvents to separate. Transferred the organic layer to a clean dry flask. Dried the solution with anhydrous sodium sulfate.

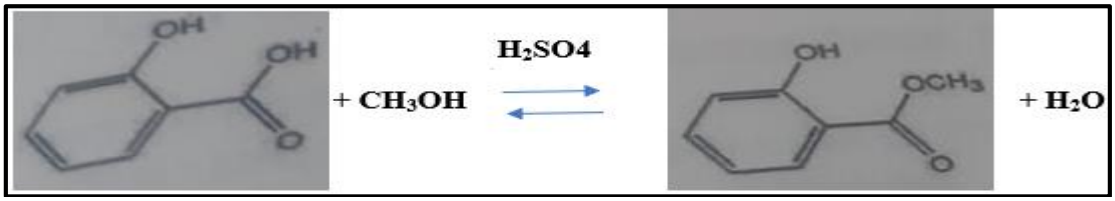

Figure 1: Synthesis scheme for Methyl salicylate

FT-IR Analysis of the Samples

FT-IR Spectrophotometry was done for the identification of the drug and detection of the possible interactions of the drug with the Excipient. For the FT-IR study, solid powder samples were oven-dried; finely crushed, mixed with Potassium Bromide (1:10 ratio by weight), and pressed at 15000 PSIG to make discs. The pellets were then scanned using FT-IR Nicolet, USA6700 instrument. The spectra were recorded and interpreted.

FORMULATION OF METHYL SALICYLATE GEL

Methyl salicylate gel was prepared by dispersing 1 gm of Carbopol in a mixture of water and 1 ml of glycerol. 200 mg of methyl paraben was used as a preservative. 1ml of methyl salicylate was added. Kept the solution under the magnetic stirring until the homogeneous dispersion was formed. The dispersion was neutralized and made viscous by the addition of 0.1 ml of triethanolamine.

COX-II ENZYME INHIBITION ASSAY OF GEL OF SYNTHESIZED METHYL SALICYLATE

Reagents and Buffers

Preparation of Stock solution

A. Extract Dilutions

Different dilutions from 0-1000µg gel /ml in Tris Cl buffer, pH 8.0 was prepared.

B. Arachidonic acid, 10 mM

10 mM stock (3.06 mg/ml) of arachidonic acid, sodium salt (Nu-Chek-Prep), in water was prepared and stored 0.5-ml aliquots for several months at −20°C. it was further diluted to 1mM to be used as working solution.

C. Cyclooxygenase (COX) enzyme solution, 2 mg/ml COX enzyme, preferably purified (e.g., Cayman) to 2 mg/ml in Tris-buffered saline with CHAPS (100 mM Tris·Cl, pH 7.5, 0.9% NaCl, 0.4% (w/v) 3-[(3-cholamidopropyl) dimethyl ammonio]-1-

propanesulfonate (CHAPS)) was dissolved and 1-ml of aliquots was stored at −80°C (stable for several years). Finally, it was diluted to 100U/ml to be used as working solution.

D. N,N,N',N′-Tetramethyl-p-phenylenediamine (TMPD), 17 mM

17 mM stock solution (4 mg/ml) of TMPD (Sigma) in water was prepared and stored 0.5-ml aliquots at –20°C. Finally, it was diluted to 2mM to be used as working solution.

E. Colexcib, 17 mM

Colexcib, 17 mM was prepared by using 50 mM stock of DMSO and stored 0.5-ml aliquots at –20°C. It was further dilute to 500µM to be used as working solution.

Preparation of Reagents

F. Tris/heme/phenol (THP) buffer, composition for 4 ml

400 µl 1 M Tris·Cl, pH 8.1 (100 mM final)

4 µl 100% water-saturated phenol, 1mM (1 µM final conc.)

40 µl bovine hemin chloride in DMSO, 100µM (1 µM heme final conc.)

Procedure

Sample dilutions in a range of 0-1000µg gel /ml in Buffer (TrisCl buffer, pH 8.0) were prepared. Reaction components were placed in each well (as described in the Reaction Mixture Set up table) of a 96-well plate. The reaction was initiated by adding 5 µl substrate and 5 µl TMPD solution and then plate was incubated at room temperature for 10 minutes and absorbance was taken at 595 nm using a micro plate reader (iMark, BioRad).

Table 1: Group wise sample dilution

	Buffer	Sample	Inhibitor	COX II	THP Buffer	Substrate	TMPD
Group 1	5µl			10	80	5	5
Group 2	10µl			10	80		5
Group 3		5		10	80	5	5
Group 4	5µl	5		10	80		5
Group 5			5	10	80	5	5
Group 6	5		5	10	80		5

RESULTS AND DISCUSSION

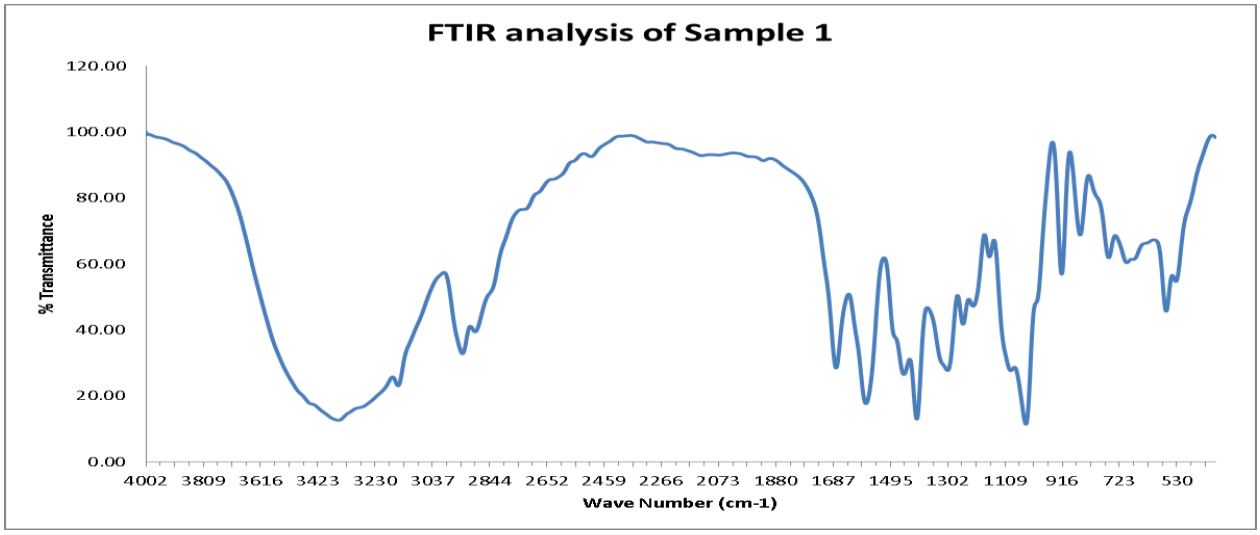

Figure 2: FTIR peak of synthesized MS

Table 2: List of FTIR Peaks of synthesized MS

S. No.	Wave Number (cm^{-1})	% Transmittance
1	3396	14.60
2	3192	23.03
3	2951	36.29
4	2920	38.49
5	1684	30.70
6	1581	18.30
7	1460	29.75
8	1412	15.98
9	1325	30.60
10	1281	41.84
11	1236	48.13
12	1188	62.68

13	1101	30.29
14	1047	14.85
15	924	62.01
16	866	74.04
17	768	64.79
18	712	63.00
19	571	47.58
20	553	53.30

Table 3: Cox II enzyme Inhibition Assay of MS Gel: Concentration of test replicates vs blank

Sample Conc.	**Test Replicates**				**Blank**		**Corrected Values**			
0	0.117	0.112	0.112	0.123	0.111	0.105	0.009	0.004	0.004	0.015
1	0.115	0.113	0.12	0.112	0.111	0.112	0.0035	0.0015	0.0085	0.0005
10	0.109	0.117	0.112	0.114	0.11	0.113	-0.0025	0.0055	0.0005	0.0025
100	0.113	0.11	0.116	0.113	0.114	0.11	0.001	-0.002	0.004	0.001
250	0.109	0.108	0.114	0.107	0.108	0.11	0.00	-0.001	0.005	-0.002
500	0.114	0.111	0.112	0.11	0.112	0.11	0.003	0.00	0.001	-0.001
1000	0.113	0.111	0.106	0.102	0.107	0.108	0.0055	0.0035	-0.0015	-0.0055
PC	0.112	0.104	0.112	0.108	0.106	0.11	0.004	-0.004	0.004	0.00

Table 4: Cox II enzyme Inhibition Assay of MS Gel: Final Values for Analysis

	Blank	**Control**					
Average Values	0	0.008					
Final Values for Analysis							
	Final Replicate Values				**Statistical data**			
Sample Conc.	**1**	**2**	**3**	**4**	**Mean**	**SD**	**SEM**	**N**
0	-12.5	50	50	-87.5	**0**	2.9734	1.7167	3
1	56.25	81.25	-6.25	93.75	**56.25**	3.4065	1.9667	3
10	131.25	31.25	93.75	68.75	**81.25**	2.2301	1.2875	3
100	87.5	125	50	87.5	**87.50**	11.682	6.7451	3
250	100	112.5	37.5	125.00	**93.75**	1.4867	0.8583	3
500	62.5	100	87.5	112.5	**90.62**	5.9469	3.4334	3
1000	31.25	56.25	118.75	168.75	**93.75**	9.8338	5.6775	3
PC	50	150	50	100	**87.5**	9.8338	5.6775	3

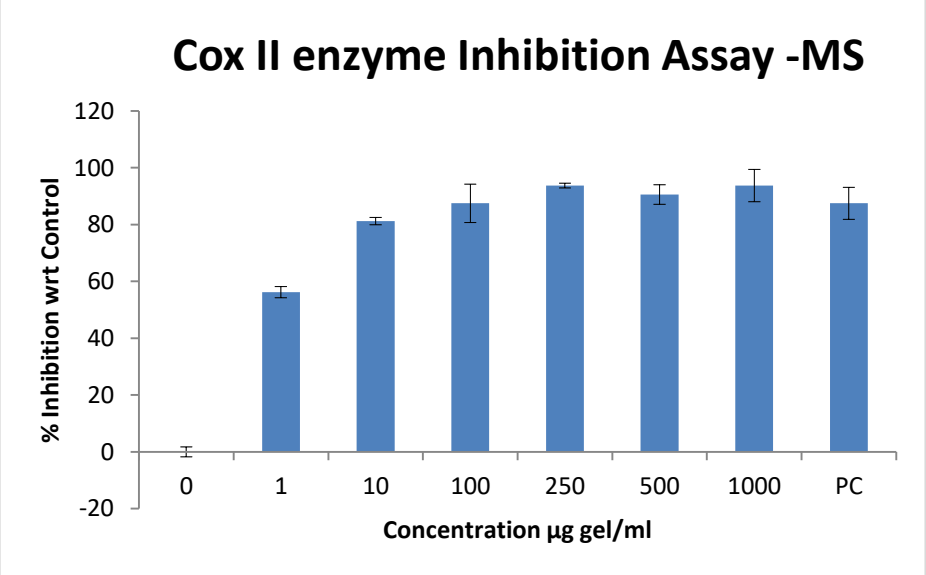

Figure 3: COX II enzyme inhibition assay of methyl salicylate

Table 5: Cox II enzyme Inhibition Assay of MS Gel: Log (inhibitor) vs. normalized response and variable slope

Best-fit values		**Std. Error**
LogIC50	-0.3658	0.1639
Hill Slope	0.3861	0.04552
IC50	**0.4307**	
95% Confidence Intervals		
Log IC50		-0.8209 to 0.08930
Hill Slope		0.2598 to 0.5125
IC50		**0.1510 to 1.228**
Goodness of Fit		
Degrees of Freedom		4
R square		0.9626
Absolute Sum of Squares		38.33
Sy.x		3.096
Number of points Analysed		6

Yield of methyl salicylate was 81% with R.F. value 0.67.

FT-IR Spectrum (Fig. 2 and table 2) of pure methyl salicylate was analyzed, the major peaks were obtained at 2920-3396, 1684, 1581, 1460, 1412, 1325, 1281, 1236, 1188, 1101, 1047,924, 866,768,712 cm^{-1}.

Whereas due to the O-H stretching vibrations it was found at 3192 to 3396 cm^{-1}, C-H stretching vibrations peaking at 2951 cm-1. The peak at ~2951 and 2920 cm^{-1} corresponds to the C-H stretching vibrations of the CH_3 group. The very characteristic carbonyl C=O stretching vibrations peaks at 1684 cm^{-1}. There are several peaks due to C-O stretching vibrations around 1325 to 1236 and 1188 to 1101 cm^{-1} (where the O is directly bonded to the benzene ring as in a phenol). There is also a C-O stretching vibration at 1188 cm^{-1} for the C-O of the ester linkage C=C stretching vibration absorptions of the benzene aromatic ring at ~1581 - 1412 cm^{-1}. The peak at 866, 768 and 712 cm^{-1} corresponds to the C-H stretching vibrations of the benzene group.

From the above all data, it can be concluded the following points. The IR spectra of MS was compared with that of the standard peak available in earlier reported articles [16-18] and identified as MS.

Cox II enzyme inhibition assay of MS Gel is tabulated in table 3, in which various concentration of test replicates vs blank were mentioned with all the corrected values. Whereas, Cox II enzyme inhibition Assay of MS Gel is tabulated in table 4, in which final values for analysis with statistical data were mentioned. COX II enzyme inhibition assay of methyl salicylate was represented in figure 3, which was plotted against the concentration of MS in the gel (mcg/ml) vs % inhibition with respect to control. It has been seen that as the concentration was increased gradually % inhibition was also increased concurrently and the values were maximum at the concentration of 1000mcg/ml. But these maximum % inhibition values were similar at the concentration of 250mcg/ml and it was approximately 94%. Although the values of % inhibition at the concentration of 500mcg/ml was little bit lower than the values at 250 and 1000, but it was greater than the PC. From this table it can be concluded that 250mcg/ml will be best among all the COX II enzyme inhibition assay of methyl salicylate.

Cox II enzyme Inhibition Assay of MS Gel is tabulated in table 5, in which it was mentioned the Log (inhibitor) vs. normalized response and variable slope with 95% Confidence Intervals of IC50 values and it was in the range of 0.1510 to 1.228, but best fit value of IC50 was 0.4307. Goodness of Fit value was statistically evaluated and also calculated in which R2 value was 0.9626, which itself prove that it was most linear and within the acceptable limit.

CONCLUSION:

From the above all data, it can be concluded the following points. The IR spectra of MS was compared with that of the standard peak available in earlier reported articles and identified as MS. But these maximum % inhibition values were similar at the concentration of 250mcg/ml and it was approximately 94%. Although the values of % inhibition at the concentration of 500mcg/ml was little bit lower than the values at 250 and 1000, but it was greater than the PC. From this table it can be concluded that 250mcg/ml will be best among all the COX II enzyme inhibition assay of methyl salicylate. Cox II enzyme inhibition assay of MS Gel is tabulated in table 5, in which it was mentioned the Log (inhibitor) vs. normalized response and variable slope with 95% Confidence Intervals of IC50 values and it was in the range of 0.1510 to 1.228, but best fit value of IC50 was 0.4307. Goodness of Fit value was statistically evaluated and also calculated in which R2 value was 0.9626, which itself prove that it was most linear and within the acceptable limit. Finally, it can be concluded that MS gel having sufficient COX II enzyme inhibition properties and which may be beneficial as compared to the others having similar to them.

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