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Kritika Soni
Research Associate, School of Medical & Allied Sciences, GD Goenka University, Gurgaon, Haryana, India

Dr. Uma Bhandari
Head of Department, Department of Pharmacology, Jamia Hamdard, New Delhi, India

Correspondence
Kritika Soni
Research Associate, School of Medical & Allied Sciences, GD Goenka University, Gurgaon, Haryana, India

Explore the mechanism of action of cinnamaldehyde against *Providencia rettgeri*: Induced alteration in choline metabolism in C57BL/6J mice

Kritika Soni and Dr. Uma Bhandari

Abstract
Atherosclerotic cardiovascular disease (ACD) is the leading cause of mortality worldwide. The present research was designed to assess the antimicrobial activity of Cinnamaldehyde using *Providencia rettgeri* and anti-atherosclerotic potential of cinnamaldehyde in choline - fed C57BL/6J mice. Molecular Docking was done to assess the protein binding ability of cinnamaldehyde with FMO3 enzyme. From molecular docking it was concluded that cinnamaldehyde inhibited the FMO3 enzyme which catalyses the conversion of TMA to TMAO, thus reduced the risk of atherosclerosis. Antimicrobial effects of cinnamaldehyde, cinnamon oil and ciprofloxacin were demonstrated by turbidimetric method. Inhibition of bacteria by test drug i.e. cinnamaldehyde and standard drug i.e. ciprofloxacin was found to be significant ($p<0.01$), so both cinnamaldehyde and ciprofloxacin showed anti-microbial activity against *P. Rettgeri*. Cinnamon oil too showed anti-microbial activity but at very low concentrations, this may be because of immiscibility of cinnamon oil in nutrient broth. From HPTLC analysis it was concluded that cinnamon oil and ciprofloxacin showed significant inhibition of *P. rettgeri*, TMA formation was inhibited. Cinnamaldehyde too showed inhibition of *P. Rettgeri* but at high concentrations. Mice fed with cinnamaldehyde (35mg/kg), cinnamon oil (46mg/kg), ciprofloxacin (100mg/kg) along with 2% choline diet each group for 21 days. Anti – atherosclerotic potential of cinnamaldehyde were demonstrated by significant reduction ($p<0.001$) in total cholesterol levels, triglyceride levels, low density lipoproteins(LDL), very low density lipoproteins (VLDL), Atherogenic risk Predictor(ARP) and coronary risk index (CRI) levels, while elevation in High – density lipoprotein (HDL) levels. Summarized together, our data suggest that cinnamaldehyde possess significant antimicrobial activity, also possess anti-atherosclerotic potential possibly by improvement in total cholesterol levels, triglyceride levels and enhancement in HDL levels.

Keywords: *Providencia rettgeri* Cinnamaldehyde FMO3 enzyme Antimicrobial HPTLC

Introduction
Atherosclerotic cardiovascular disease (ACD) is the leading cause of mortality worldwide. Cardiovascular mortality decreased globally from 1990-2010 (Baquera *et al,* 2015) [1]. According to a recent World Health Organization (WHO) Report, more than 82% deaths due to cardiovascular disease (CVD) have occurred in developing countries. The number of deaths due to CVDs has been estimated to reach beyond 23 million by 2030 (Mendis *et al,* 2011) [21]. The complex interplay of genetics, nutrition and environmental factors leading to atherosclerosis continues to attract great investigation. One well known risk factor in humans is hypercholesterolemia and other important contributors to this disease include inflammation, oxidative stress and insulin resistance. Hyperlipidemia contributes significantly to the manifestation and development of atherosclerosis and coronary artery disease. Foods rich in saturated fats and cholesterol have been linked to circulating cholesterol levels.

A very little is known about the role of phospholipids in atherosclerosis. The present study focused on phospholipids metabolism. Phosphatidylcholine (PC) are a class of phospholipids that incorporate choline as a head group and can be easily obtained from a variety of food sources such as egg yolk, peanuts, dairy products, spinach, beets, wheat, shellfish etc. Although phosphatidylcholine is present in almost all cells in the body, the highest concentration is found in the brain, heart, liver and kidney.

Phosphatidylcholine in the presence of phospholipase D converts to choline, a water soluble nutrient essential for human life and grouped within vitamin B complex. Choline is further metabolized by gut microbiota to produce an intermediate trimethylamine (TMA). TMA is quickly reabsorbed from gut lumen into host plasma and rapidly oxidized to TMAO in the

liver by hepatic flavinmonooxygenases (FMOs), particularly FMO3. Supplementation with choline or TMAO promoted atherosclerosis (Koeth *et al*, 2013) [18]. Functional studies showed that trimethylamine- N-oxide(TMAO) inhibited reverse cholesterol transport(RCT) and promoted accumulation of cholesterol in macrophages through increasing scavenger receptors, Cluster of differentiation(CD) 36 and scavenger receptor (SRA) (Wang *et al*, 2011) [32] reducing synthesis of bile acids from cholesterol, and decreasing expression of bile acid transporters in the liver (Koeth *et al*,2013) [18].

It was identified that 8 species representing 2 different phyla that is Firmicutes and Proteo bacteria and 6 genera showed significant choline consumption and TMA accumulation: *Escherichia fergusonii, Anaerococcus hydrogenalis, Clostridium hathewayi, C. sporogenes, Proteus penneri, Providencia rettgeri and Edwardsiellatarda* (Romano *et al*,2015) [29].

In the present study, *Providencia rettgeri* microbe was used. *Providencia rettgeri* is a gram negative bacteria isolated by Rettger in 1904 responsible for wide range of human infections. Broad spectrum anti-bacterial drugs that have the potential to suppress the infectious burden reduce the formation of trimethylamine -N-oxide (TMAO) from choline metabolism (Elkind, 2010) [7]. In the present study, ciprofloxacin was used as the standard drug. It is a second – generation fluoroquinolone antibiotic. It targets bacterial DNA gyrase and topoisomerase IV. These are potent bactericidal agents against a broad variety of microorganisms and have good activity against staphylococci but not against methicillin- resistant strains.

Cinnamaldehyde consists of the dried inner bark of the shoots of coppiced trees of Cinnamomum zeylanicum, family- Lauraceae. Cinnamaldehyde reduced mean fasting serum glucose, total cholesterol, low density lipoprotein cholesterol (LDL-C). It also possess anti-inflammatory and antimicrobial properties (Ooi *et al*, 2006) [25]. Cinnamaldehyde acts as a nitric oxide donor, also suppress TNF- induced signaling pathways thus, helps in preventing atherosclerosis. Cinnamaldehyde showed good antibacterial activity against Proteus spp. and Klessiella pneumonia by liquid microdilution method and decreased biofilm formation of Enterhemorrhagic E.coli (Kim *et al*, 2015) [17]. Due to these successes, there was a possibility that Cinnamaldehyde has potential for the management of atherosclerosis. None of the study of Cinnamaldehyde on choline metabolism and its role in gut microbiota has been reported.

Recent studies revealed that TMAO exacerbates atherosclerosis in mice and positively correlates with the severity of this disease in humans. Therefore, in the present study, female C57BL/6J mice were used. As such mice are highly resistant to atherosclerosis, the only exception is the C57BL/6J strain of mice (Jawein *et al*, 2011) [14].

The present research was designed to assess the antimicrobial activity of cinnamaldehyde using *Providencia rettgeri* and anti-atherosclerotic potential of Cinnamaldehyde in choline - fed C57BL/6J mice.

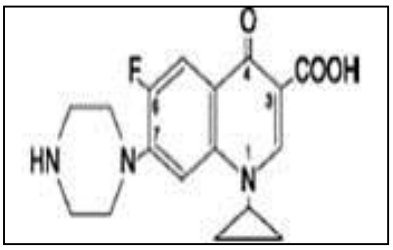

Fig 1: Chemical structure of ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3- quinolinecarboxylic acid)

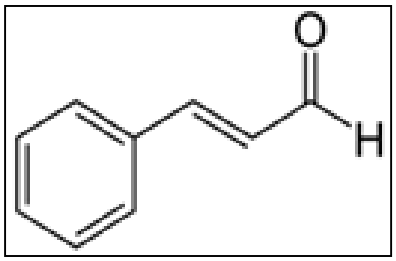

Fig 2: Chemical structure of cinnamaldehyde ((2E)-3-Phenylprop-2-enal)

Materials and Methods

Chemicals

Cinnamaldehyde was purchased from SD Fine Chemicals Pvt. Ltd., Mumbai. Ciprofloxacin was purchased from CIPLA Pvt. Ltd., Mumbai. Various standard kits were used for the investigation of anti- atherosclerotic potential of cinnamaldehyde viz. total cholesterol, triglycerides, HDL cholesterol from Span Diagnostics Ltd., Surat, India.

Molecular Docking

The molecular docking study was carried out in order to assess their interaction and binding modes with target receptor/ enzyme and good biological activity using the software Glide extra precision (XP) Maestro 10.1 Schrodinger, running on Linux 64 operating system. In molecular docking the 2D structure for synthesized compounds was generated and then converted to their respective 3D structures with use of Ligprep. The X-ray crystal structure of 2GV8 was downloaded from Protein Data Bank solved at a resolution of 2.1 angstrom. Molecular docking studies mainly involve selection and preparation of appropriate protein, grid generation, ligand preparation followed by docking and its analysis. The docking score and hydrogen bonds and pi-pi interaction formed with the surrounding amino acid are used to predict their binding affinities and proper alignment of these compounds at the active site of the receptor/enzyme (Schrodinger, Maestro, Version 10.1,LLC, New York, NY, 2016).

Cinnamon oil extraction

The Cinnamon was procured from the local market and it was authenticated by the Department of Pharmacognosy. A voucher specimen is retained in the Department of Pharmacognosy (No. 001). Cinnamon oil was extarcted from Clevenger's apparatus by hydrodistillation method (Jeyaratnam *et al*, 2016) [16]. The oil was kept in deep freezer at -20 ºC.

Microbial inhibition by cinnamon oil, cinnamaldehyde and ciprofloxacin by turbidimetric method

Before beginning the experimental procedure, all the laboratory glasswares required were sterilized by autoclaving at 15 psi at 121 degree Celsius for 15 minutes. Experiments were performed in aseptic condition. After preparation of Agar, it was poured into the autoclaved petri plates and slants and was left for solidification..After solidification of agar petri plates were sealed with parafilms and slants were cotton plugged and kept in tilt position. Petri plates and slants were taken and the solidification was checked then streaking was done in the laminar flow in all the petri plates and slants and then kept in the incubator for 24 hours.

Preparation of 7 culture tubes (Cinnamon oil) : Negative control [10 ml nutrient broth + bacteria+ water (100 microlitre)], Positive control [10ml nutrient broth+ bacteria] and five different concentrations of cinnamon oil(10, 35, 60,80,100 microlitre/10ml media) were prepared. Preparation of 7 culture tubes (Cinnamaldehyde) : Negative control [10 ml nutrient broth + bacteria+ water(100 microlitre)], Positive control [10ml nutrient broth+ bacteria] and five different concentrations of cinnamaldehyde (1,2,3,4,5microlitre/10ml media) were prepared. Preparation of 7 culture tubes (Ciprofloxacin): Negative control [10 ml nutrient broth + bacteria +water(100 microlitre)], positive control[10 ml nutrient broth+bacteria]andfivedifferentconcentrationsofciprofloxacin(10,20,30,40,50microlitre/10ml of media) these culture tubes were placed in incubator then after 24 hours optical density was measured using U.V.spectrophotometer at 610 nm.

Analysis of formation of TMA from choline in presence and absence of cinnamaldehyde, cinnamon oil and ciprofloxacin by HPTLC method.

Development of solvent system

Pre coated silica TLC plates (aluminium- backed silica plates) were used to elute the TMA.The TLC plates were activated in hot air oven at 105 °C for 15 minutes. Standard TMA was dissolved in minimum quantity of ethanol. Spot of standard TMA was applied on activated TLC plate and it was allowed to run in solvent system. Solvent system consists of n-Butanol-ethanol-acetic acid-water in ratio of 8:2:1:3. After elution the spots were observed under ultraviolet light at 254nm and 366nm.For this analysis standard plot of TMA was prepared with different serial dilution of standard TMA (50µg/ml, 100µg/ml,200µg/ml,250µg/ml). Different sets were prepared consisting of different concentrations of cinnamaldehyde(1, 2,3,4 µg/ml), different concentrations of cinnamon oil(1,3,6,10µg/ml) and different concentrations of ciprofloxacin(1,2,5,10µg/ml).

All the set were incubated at 37 °C for 24 hours. After 24 hours 5ml of media from all the sets were taken and 5ml of chloroform was added to it for extraction of TMA. The amount of TMA formed were measured using HPTLC-UV. Chromatographic system consists of Mode: HPTLC Detector: UV 254 and 366nm, Column :Twin trough chamber, Temperature : 37 °C, Injection size:100µl,Platesize:20x10cm.

Experimental animals

This study was approved by Institutional Animal Ethics Committee (IAEC) of Jamia Hamdard, New Delhi 110062, in its meeting held on 8.11.2016. C57BL/6J mice, weighing 20-30 g of either sex of 5-6 weeks were procured from the Central Animal House Facility, Hamdard University, New Delhi. The animals were kept in propylene cages under the standard laboratory conditions (12 hour light and 12 hour dark; day: night cycle and was free access to commercial pellet diet and tap water ad libitum. The animal house temperature was maintained at 25± 2 °C and relative humidity was also be maintained at (50±15%). The experiment was carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Experimental Protocol

After acclimatization, the animals were divided into six groups as follows:

Group1: Mice fed with normal pellet diet with normal saline for 21 days

Group2: Mice fed with 2% choline diet for 21days

Group 3: Mice fed with cinnamaldehyde (35mg/kg) for7 days + 2% choline diet from 8th day to 21 days

Group 4: Mice fed with 2% choline diet from 1st day to 14 days+ cinnamaldehyde (35mg/kg) from15thday to21days

Group 5: Mice fed with 2% choline diet along with cinnamon oil (46mg/kg) for 21 days

Group 6: Mice fed with 2% choline diet along with ciprofloxacin (100mg/kg) orally for 21days

After 21 days,mice were fasted overnight and next day samples of whole blood were collected in tubes for biochemical estimation. Mice were anesthetized by ether and the blood was withdrawn from retino orbital route. The blood was collected in sterile centrifuge tubes and serums were separated for biochemical estimation. The serum was aspirated using a micropipette after centrifugation at 2000 rpm for 10 minutes and was collected in Eppendroff tubes marked accordingly and was used for biochemical estimation.

Anthropogenic Parameters

Daily food supplements intake was measured by calculating the amount of food given and the left over. The body weight was measured weekly.

Biochemical estimation in serum

Assessment of total cholesterol (TC) (Siedal *et al*, 1983, Demacher *et al*, 1980) [5]: Total cholesterol levels were measured using the commercially available standard kit and according to manufacturer's instructions using the formula:

$$\text{Total cholesterol (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} X200$$

Assessment of Triglycerides (TGs) (Stein *et al*, 1995, Foster and dunn, 1973): Total triglycerides levels were measured using the commercially available standard kit and according to manufacturer's instructions using the formula:

$$\text{Triglycerides (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \text{X } 200$$

Assessment of high density lipoprotein- cholesterol (HDL-C) (Siedal *et al*, 1983): Total HDL-C levels were also measured using the commercially available standard kit and according to manufacturer's instructions using the formula:

$$\text{Serum HDL-cholesterol (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \text{X } 50$$

Assessment of low density lipoprotein-cholesterol (LDL-C) (Friedewald *et al*, 1972) [8]
Serum LDL-C level was determined according to the method of (Friedwald *et al*,1972) [8]

$$\text{LDL-cholesterol} = \text{Total cholesterol} - \frac{\text{Triglycerides}}{5} - \text{HDL-cholesterol}$$

Assessment of very low density lipoprotein- cholesterol (VLDL- C) (Friedewald *et al*, 1972) [8]
Serum VLDL-C level was determined according to the method of Friedwald *et al*, 1972 [8]

$$\text{VLDL-Cholesterol} = \frac{\text{Triglycerides}}{5}$$

Assessment of atherogenic risk predictor indices (Dobiasova and Frohlich, 2001) [6]
Coronary risk index (CRI)
CRI=Total cholesterol/HDL-Cholesterol
Atherogenic risk predictor (ARP)
ARP = LDL-Cholesterol/ HDL-Cholesterol

Statistical Analysis
Statistical Analysis was carried out using GraphpadInstat (Graphpad software: San Diago, CA). All results were expressed as Mean±S.E.M. Groups of data were compared with an analysis of variance followed by Tukey t-test.

Results
Molecular Docking
From molecular docking it was observed that the design molecule (cinnamaldehyde) was capable of inhibiting FMO3 enzyme. Cinnamaldehyde was better than ciprofloxacin in inhibiting FMO3 enzyme thus, inhibiting formation of TMAO and reduces the risk of atherosclerosis, because it has docking score (-8.671 Kcal/mol) as compared to ciprofloxacin (-5.313 Kcal/mol). Cinnamaldehyde had more negative value than ciprofloxacin; more negative value means more stability to bind to the receptor site.

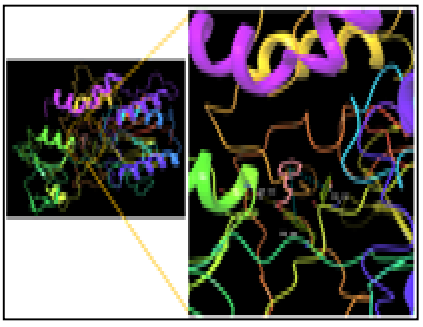

Fig 3

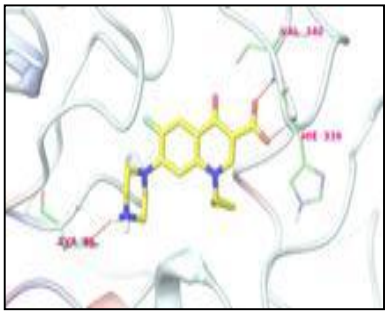

Fig 4

Microbial inhibition by cinnamon oil, cinnamaldehyde and ciprofloxacin against *Providencia rettgeri* by turbidimetric method
With increase in% concentration of Cinnamon oil, there was decrease in% inhibition of bacteria (*Providencia rettgeri*)
Maximum antibacterial inhibition was 86.7% with 0.1% concentration of cinnamon oil (Table 1, Figure 5)
With the increase in% concentration of Cinnamaldehyde, there was increase in% inhibition of bacteria (*Providencia rettgeri*).
Maximum antibacterial inhibition was 89.3% with 0.05% concentration of cinnamaldehyde (Table 2, Figure 6)
With the increase in% concentration of Ciprofloxacin there was increase in% inhibition of bacteria (*Providencia rettgeri*).
Maximum antibacterial inhibition was 83.8% with 0.5% concentration of ciprofloxacin (Table 3, Figure 7)

Table 1: %age concentration of cinnamon oil and%age inhibition of bacteria.

% concentration of Cinnamon oil	% inhibition of bacteria
0.1	86.7
0.35	77
0.6	58.8
0.8	56.71
1	53.63

Table 1:%age concentration of cinnamon oil and%age inhibition of bacteria

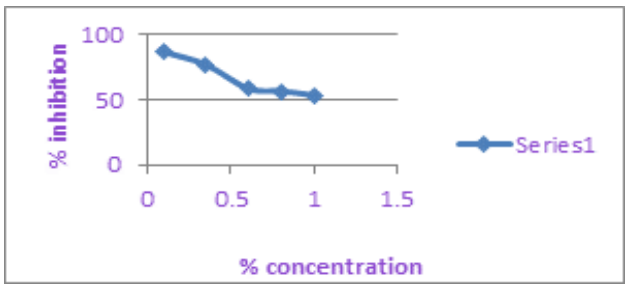

Fig 5: Plot of%age concentration of cinnamon oil and%age inhibition of bacteria

Table 2: %age concentration of cinnamaldehyde and%age inhibition of bacteria

% Concentration of Cinnamaldehyde	% inhibition of bacteria(*P. rettgeri*)
0.01	77.4
0.02	82.7
0.03	85.8
0.04	87.2
0.05	89.3

Table 2: %age concentration of cinnamaldehyde and%age inhibition of bacteria

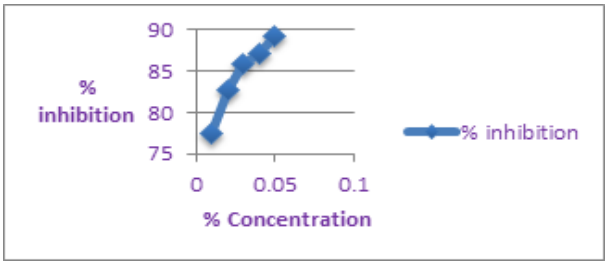

Fig 6: Plot between%age concentration of cinnamaldehyde and%age inhibition of bacteria

Table 3: concentration of ciprofloxacin and% inhibition of bacteria

%concentration of ciprofloxacin	% inhibition of bacteria(*Providenciarettgeri*)
0.1	69.1
0.2	73.5
0.3	78.3
0.4	82.8
0.5	83.8

Table 3: concentration of ciprofloxacin and%age inhibition of bacteria

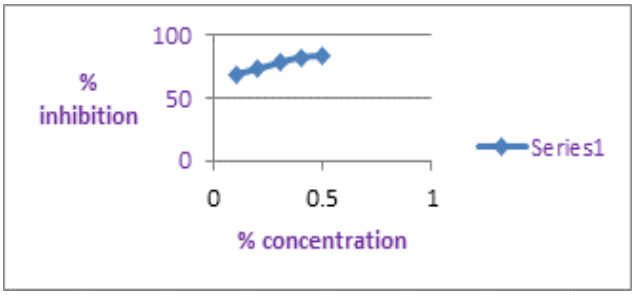

Fig 7: Plot between%age concentration of ciprofloxacin and%age inhibition of bacteria

Analysis of TMA formed from choline by *Providencia rettgeri* in presence and absence of drug by HPTLC method

The reaction that is conversion of choline to trimethylamine (TMA) in vitro in presence of *Providencia rettgeri* was carried out. The concentration of TMA (µg/ ml) in various samples was calculated using HPTLC. It was found that the test drug i.e. Cinnamaldehyde (1, 2, 3 µg / ml) at these concentrations choline was metabolized to TMA. At 4 µg / ml concentration the choline was not metabolised to TMA. Thus at 4 µg / ml concentration Cinnamaldehyde inhibited bacteria. TMA formation was inhibited. Cinnamon oil showed significant inhibition of *P. rettgeri* as choline was not metabolised to TMA. TMA formation was inhibited. The standard drug Ciprofloxacin also showed significant inhibition of *P. rettgeri* as TMA formation was inhibited. Cinnamon oil and Ciprofloxacin both were actively inhibiting Choline to TMA conversion.

Table 4: Different dilutions of standard TMA and their respective peak area

S.NO.	Concentration of Standard TMA(µg/ml)	Peak Area(AU)
1.	250	1764.5
2.	200	1654.6
3.	100	1129.7
4.	50	864.5

Table 4: Different dilutions of standard TMA and their respective peak area

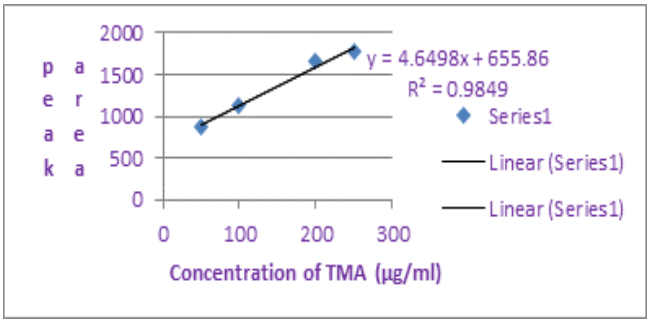

Fig 8: Standard plot of TMA (concentration vs. peak area)

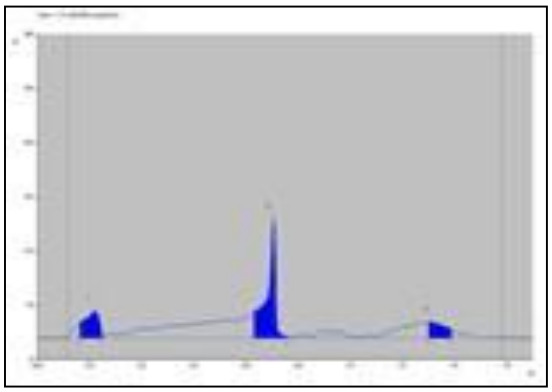

Fig 9: Chromatograms of Standard TMA (250µg/ml)

Table 5: Concentration of TMA (µg/ml) in different samples as calculated from the standard plot of HPTLC chromatogram

Sample No	Sample Name	Concentration of TMA (µg/ml)
1	Nutrient Broth	-110.22
2	Nutrient Broth +*P. rettgeri*	-174.00
3	Broth +*P. rettgeri* +Choline	-177.80
4	Broth + *P. rettgeri* + Choline+1µg/ml Cinnamaldehyde	115.65
5	Broth +*P. rettgeri* + Choline + 2 µg/ml Cinnamaldehyde	112.17
6	Broth +*P. rettgeri* + Choline + 3 µg/mlCinnamaldehyde	107.43
7	Broth+*P. rettgeri* + Choline + 4 µg/ml Cinnamaldehyde	-111.17
8	Broth +*P. rettgeri* + Choline+ 1 µg/ml cinnamon oil	-154.34
9	Broth + *P. rettgeri* + Choline+ 2µg/ml cinnamon oil	-199.32
10	Broth+*P. rettgeri* + Choline + 4µg/ml of cinnamonoil	-222.71
11	Broth +*P. rettgeri* + Choline+ 10µg/ml of cinnamon oil	-168.44
12	Broth +*P. rettgeri* + Choline + 1µg/ml of Ciprofloxacin	-188.74
13	Broth +*P.rettgeri* + Choline + 2 µg/ml of Ciprofloxacin	-185.80
14	Broth+ *P.rettgeri* + Choline + 3 µg/ml of Ciprofloxacin	-182.2
15	Broth+ *P.rettgeri* + Choline + 10µg/ml of Ciprofloxacin	-170.02

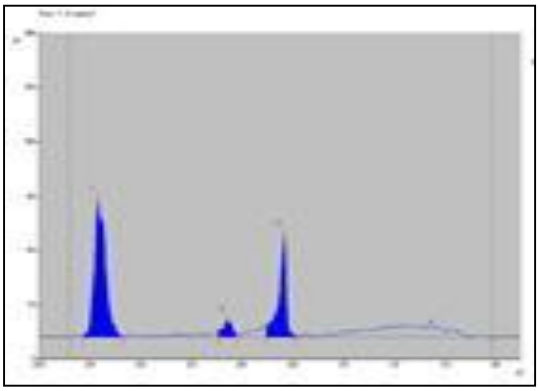

Fig 10: Chromatogram of Cinnamaldehyde

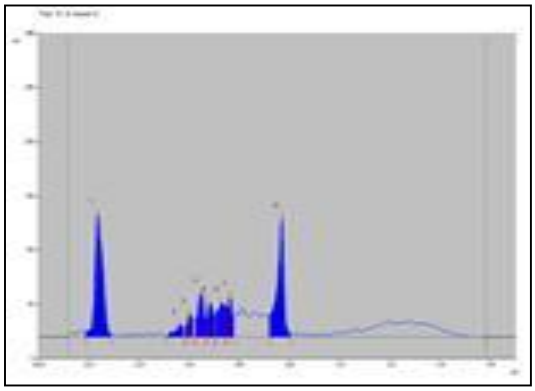

Fig 11: Chromatogram of Ciprofloxacin Effect on daily food intake (g/mice/day)

There was significant increase (p<0.001) in mean daily food intake in cinnamaldehyde (35mg/kg) treated group, cinnamon oil (46mg/kg) treated group and ciprofloxacin (100mg/kg) treated group as compared to toxic control mice.

Table 6: Effect of Cinnamaldehyde on daily food intake in C57BL/6J mice

Group No.	Treatment	g/mice/day
I	Normal control	19.4 ± 0.245
II	Toxic control	12.7 ± 0.588***
III	Cinnamaldehyde(CA) (35mg/kg) preventive study	19.3 ± 0.686***
IV	Cinnamaldehyde(CA) (35mg/kg) curative study	16.6 ± 0.615***
V	Cinnamon oil(46mg/kg)	15.3 ± 1.92***
VI	Ciprofloxacin(CFX) (100mg/kg)	11.5 ± 2.36**

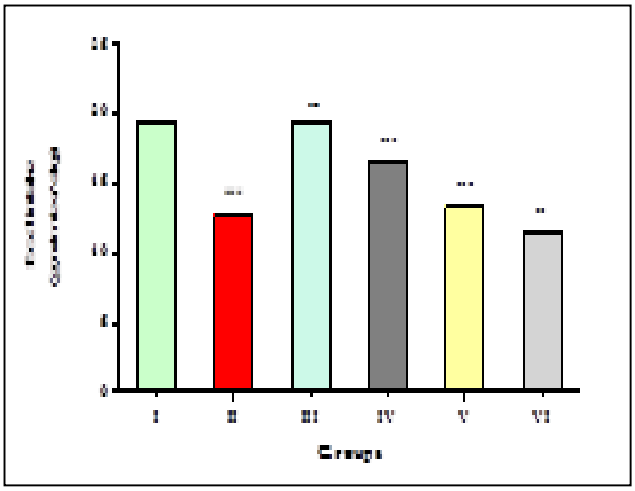

Fig 12: Effect of Cinnamaldehyde on daily food intake in C57BL/6J mice Effect on daily water intake (ml/mice/day)

There was significant increase (p<0.01) in mean daily water intake in cinnamaldehyde (35mg/kg) treated group, there was no significant increase (p>0.05) in cinnamon oil (46mg/kg) treated group and ciprofloxacin (100mg/kg) treated group as compared to toxic control mice.

Table 7: Effect of Cinnamaldehyde on daily water intake in C57BL/6J mice

Group No.	Treatment	ml/mice/day
I	Normal control	75.2 ± 2.36
II	Toxic control	77.2± 0.875
III	Cinnamaldehyde(CA) (35mg/kg) preventive study	79.8± 0.945
IV	Cinnamaldehyde(CA) (35mg/kg) curative study	78.6± 0.706
V	Cinnamon oil(46mg/kg)	77.6± 1.28
VI	Ciprofloxacin(CFX) (100mg/kg)	78.0± 1.03

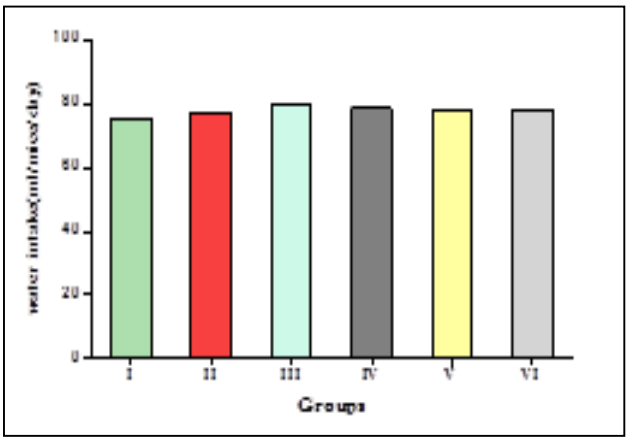

Fig 13: Effect of Cinnamaldehyde on daily water intake in C57BL/6J mice Effect on body weight gain (g/mice/day)

Effect on body weight gain (g/mice/day)

There was significant increase (p<0.001) in body weight in cinnamaldehyde (35mg/kg) treated group, there was decrease (p<0.05) in cinnamon oil (46mg/kg) treated group and there was no significant decrease in ciprofloxacin (100mg/kg) treated group as compared to toxic control mice.

Table 8: Effect of Cinnamaldehyde on Body weight gain in C57BL/6J Mice

Group No.	Treatment	g/mice/day
I	Normal control	19.9 ± 0.0576
II	Toxic control	18.9 ± 0.115***
III	Cinnamaldehyde(CA) (35mg/kg) preventive study	19.9 ± 0.124**
IV	Cinnamaldehyde(CA) (35mg/kg) curative study	20.4± 0.177***
V	Cinnamon oil(46mg/kg)	17.9 ± 0.323**
VI	Ciprofloxacin(CFX) (100mg/kg)	18.2 ± 0.255**

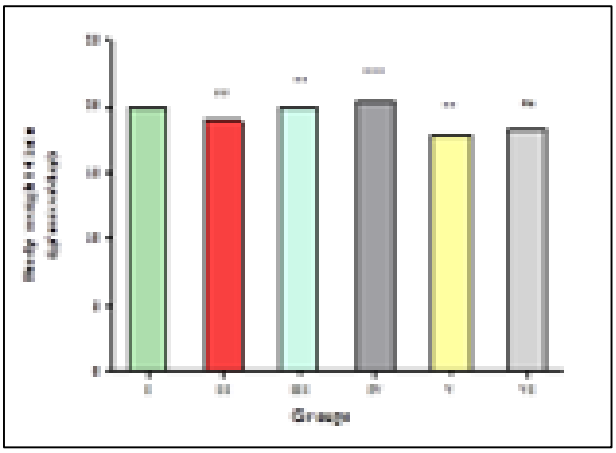

Fig 14: Effect of Cinnamaldehyde on Body weight gain in C57BL/6J Mice Effect of cinnamaldehyde on total cholesterol

There was significant decrease (p<0.001) in cholesterol levels in cinnamaldehyde (35mg/kg) treated group, cinnamon oil (46mg/kg) treated group, there was no significant decrease (p>0.05) in ciprofloxacin (100mg/kg) treated group as compared to toxic control mice.

Table 9: Effect of Cinnamaldehyde on Total Cholesterol in C57BL/6J mice

Group Number	Group Name	Total Cholesterol (mg/dl)
I	Normal Control	109 + 2.83
II	Toxic Group	171 + 0.912###
III	Cinnamaldehyde(CA) (35mg/kg):preventive study	135 + 2.11***
IV	Cinnamaldehyde(CA) (35mg/kg):curative study	121 + 2.07***
V	Cinnamon oil(46mg/kg)	119 + 1.65***
VI	Ciprofloxacin(CFX) (100mg/kg)	177 + 2.72ns

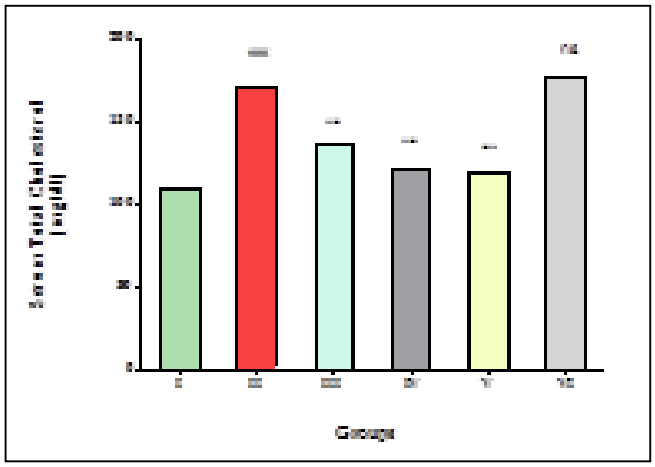

Fig 15: Effect of Cinnamaldehyde on Total Cholesterol in C57BL/6J mice Effect of cinnamaldehyde on triglyceride levels

There was significant decrease (p<0.01) in triglyceride levels in cinnamaldehyde (35mg/kg) treated group, cinnamon oil (p<0.001) (46mg/kg) treated group, there was no significant decrease (p>0.05) in ciprofloxacin (100mg/kg) treated group as compared to toxic control mice.

Table 10: Effect of Cinnamaldehyde on HDL-Cholesterol in C57BL/6J mice

Group Number	Group Name	Triglycerides (mg/dl)
I	Normal Control	69.3 ± 1.05
II	Toxic Group	90.2 ± 2.58***
III	Cinnamaldehyde(CA) (35mg/kg):preventive study	80.5 ± 1.31**
IV	Cinnamaldehyde(CA) (35mg/kg):curative study	78.8 ± 1.05**
V	Cinnamon oil(46mg/kg)	73.5 ± 1.30***
VI	Ciprofloxacin(CFX) (100mg/kg)	87.6 ± 2.52**

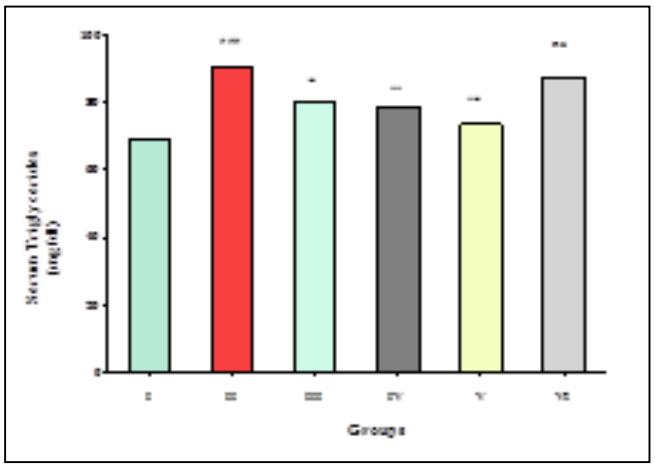

Fig 16: Effect of Cinnamaldehyde on Triglycerides in C57BL/6J mice Effect of cinnamaldehyde on HDL levels

There was significant increase (p<0.001) in HDL levels in cinnamaldehyde (35mg/kg) treated group, cinnamon oil (p<0.001) (46mg/kg) treated group, there was also increase (p<0.01) in ciprofloxacin (100mg/kg) treated group as compared to toxic control mice.

Group Number	Group Name	HDL Cholesterol (mg/dl)
I	Normal Control	97.5 ± 1.05
II	Toxic Group	68.7 ± 1.11***
III	Cinnamaldehyde(CA) (35mg/kg):preventive study	118 ± 0.987***
IV	Cinnamaldehyde(CA) (35mg/kg):curative study	119 ± 1.10***
V	Cinnamon oil(46mg/kg)	118 ± 1.04***
VI	Ciprofloxacin (CFX) (100mg/kg)	78.1 ± 0.865***

Table 11: Effect of Cinnamaldehyde on Triglycerides in C57BL/6J mice.

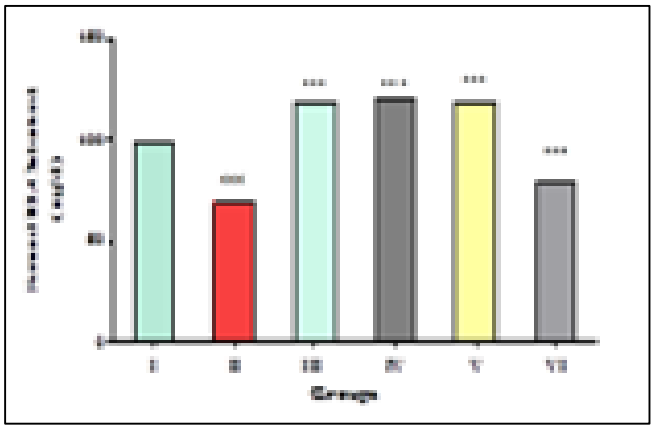

Fig 17: Effect of Cinnamaldehyde on HDL-Cholesterol in C57BL/6J mice Effect of cinnamaldehyde on LDL levels

There was significant decrease (p<0.001) in LDL levels in cinnamaldehyde (35mg/kg) treated group, cinnamon oil (p<0.001) (46mg/kg) treated group, there was no significant decrease (p>0.05) in ciprofloxacin (100mg/kg) treated group as compared to toxic control mice.

Table 12: Effect of Cinnamaldehyde on LDL-Cholesterol in C57BL/6J mice

Group Number	Group Name	LDL-Cholesterol
I	Normal Control	3.52 ± 1.06
II	Toxic Group	84.7 ± 2.24***
III	Cinnamaldehyde(CA) (35mg/kg):preventive study	4.65 ± 1.61***
IV	Cinnamaldehyde(CA) (35mg/kg):curative study	14.0 ± 1.92***
V	Cinnamon oil(46mg/kg)	14.2 ± 2.25***
VI	Ciprofloxacin(CFX) (100mg/kg)	81.2 ± 2.23**

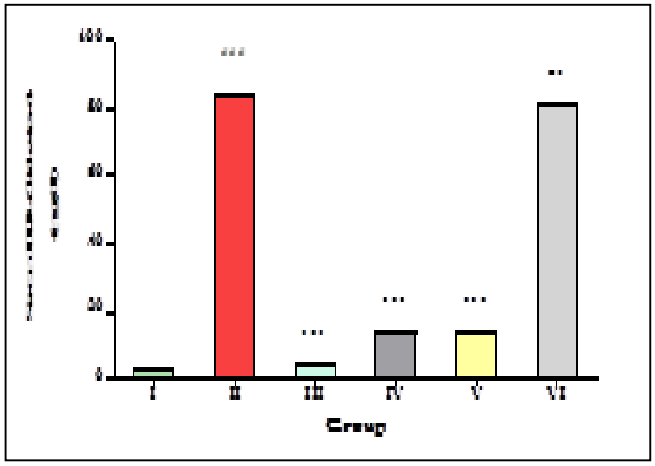

Fig 18: Effect of Cinnamaldehyde on LDL-Cholesterol in C57BL/6J mice Effect of cinnamaldehyde on VLDL levels

There was significant decrease (p<0.01) in V LDL levels in cinnamaldehyde (35mg/kg) treated group, cinnamon oil (p<0.001) (46mg/kg) treated group, there was no significant decrease (p>0.05) in ciprofloxacin (100mg/kg) treated group as compared to toxic control mice.

Table 13: Effect of Cinnamaldehyde on VLDL-Cholesterol in C57BL/6J mice

Group Number	Group Name	VLDL- Cholesterol (mg/dl)
I	Normal Control	13.9 ± 0.210
II	Toxic Group	18.0 ± 0.516***
III	Cinnamaldehyde(CA) (35mg/kg):preventive study	16.1 ± 0.261**
IV	Cinnamaldehyde(CA) (35mg/kg):curative study	15.8 ± 0.211**
V	Cinnamon oil(46mg/kg)	14.7 ± 0.260***
VI	Ciprofloxacin(CFX) (100mg/kg)	17.5 ± 0.504**

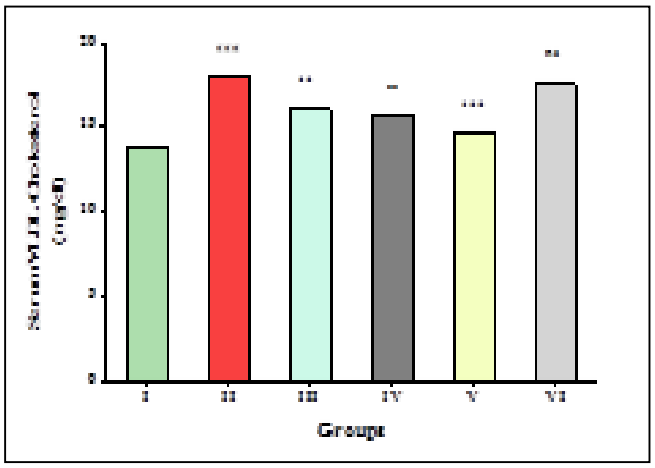

Fig 19: Effect of Cinnamaldehyde on VLDL-Cholesterol in C57BL/6J mice Effect of cinnamaldehyde on ARP levels

There was significant decrease (p<0.001) in ARP levels in cinnamaldehyde (35mg/kg) treated group, cinnamon oil (p<0.001) (46mg/kg) treated group, there was no significant decrease (p>0.05) in ciprofloxacin (100mg/kg) treated group as compared to toxic control mice.

Table 14: Effect of Cinnamaldehyde on ARP value in C57BL/6J mice

Group Number	Group Name	Atherogenic Risk Predictor
I	Normal Control	0.0336 ± 0.0102
II	Toxic Group	1.23 ± 0.0526***
III	Cinnamaldehyde(CA) (35mg/kg):preventive study	0.038 ± 0.0137***
IV	Cinnamaldehdye(CA) (35mg/kg):curative study	0.115 ± 0.0161***
V	Cinnamon oil(46mg/kg)	0.118 ± 0.0183***
VI	Ciprofloxacin(CFX) (100mg/kg)	1.14 ± 0.103**

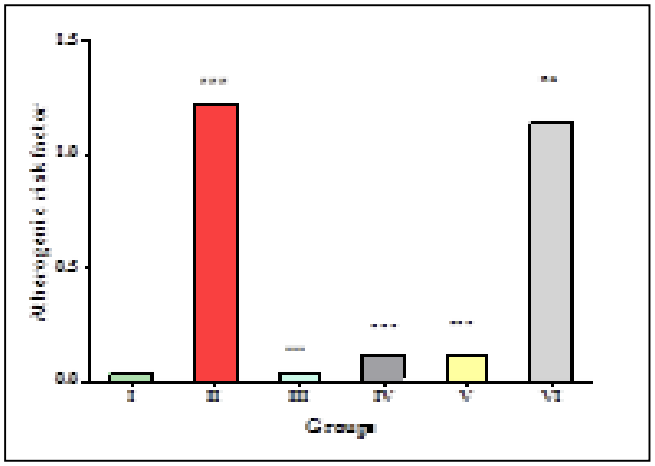

Fig 20: Effect of Cinnamaldehyde on ARP value in C57BL/6J mice Effect of cinnamaldehyde on CRI levels

There was significant decrease (p<0.001) in CRI levels in cinnamaldehyde (35mg/kg) treated group, cinnamon oil (p<0.001) (46mg/kg) treated group, there was decrease (p<0.01) in ciprofloxacin (100mg/kg) treated group as compared to toxic control mice.

Group Number	Group Name	Coronary Risk Index
I	Normal Control	1.12 ± 0.019
II	Toxic group	2.50 ± 0.051***
III	Cinnamaldehdye(CA) (35mg/kg) preventive study	1.14 ±0.023***
IV	Cinnamaldehye(CA) (35mg/kg) curative study	1.01 ±0.014***
V	Cinnamon oil(46mg/kg)	1.00 ±0.021***
VI	Ciprofloxacin(CFX) (100mg/kg)	2.31 ±0.038***

Table 15: Effect of Cinnamaldehyde on CRI values in C57BL/6J mice

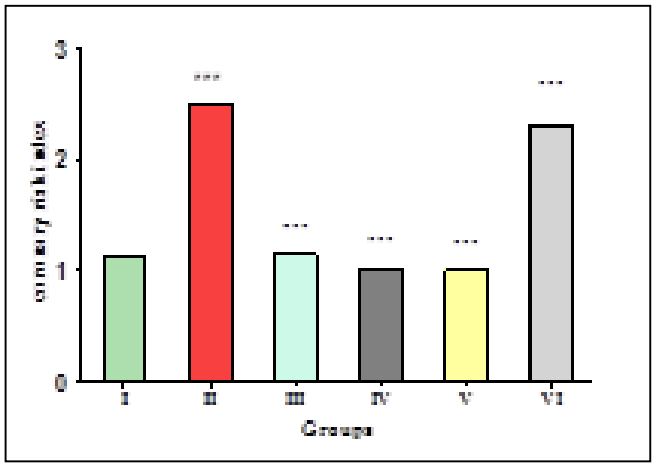

Fig 21: Effect of Cinnamaldehyde on CRI values in C57BL/6J mice

Discussion

There are number of herbal drugs available used for the treatment of atherosclerosis such as *Allium sativum*, *Aeglemarmelos*, *Curcuma longa*, *Cinnamomum cassia etc.* Among these drugs cinnamaldehyde, the main constituent of cinnamon has attracted attention due to its ability to exert beneficial effects in multiple pathological conditions.

Cinnamaldehyde reduced mean fasting serum glucose, total cholesterol, low density lipoprotein cholesterol (LDL-C). It also possess anti-inflammatory and antimicrobial properties. Cinnamaldehyde acts as a Nitric oxide donor, also suppress TNF- induced signaling pathways thus helps in preventing atherosclerosis. Cinnamaldehyde showed good antibacterial activity against *Proteus spp.* and *Klessiella pneumonia* by liquid microdilution method and decreased biofilm formation of EnterhemorrhagicE.coli (Kim *et al*, 2015) [17].

At present no research has been done to investigate the effect of Cinnamaldehyde on choline metabolism. The present study was an attempt to find out the effectiveness of Cinnamaldehyde for reducing TMA and TMAO level.

In the present study, the effects of Cinnamaldehyde on choline metabolism by *in-silico*, *in-vitro* and *in-vivo* methods were investigated. The *in silico*test was done to assess the protein binding ability of cinnamaldehyde with FMO3 enzyme Cinnamaldehyde inhibited the FMO3 enzyme which catalyses the conversion of TMA to TMAO, thus reducing the risk of atherosclerosis.

Here, the *in- vitro* comparative analysis of cinnamon oil and cinnamaldehyde was carried out to assess the antimicrobial activity against *P. rettgeri* and concentration of trimethylamine (TMA) formed from choline in presence and absence of Cinnamaldehyde was examined.

Anti- microbial effects of cinnamon oil, cinnamaldehyde and ciprofloxacin was evaluated by Turbidimetric method. With increase in% concentration of cinnamon oil, there was decrease in% inhibition of bacteria (*Providencia rettgeri).*Maximum antibacterial inhibition was 86.7% with 0.1% concentration of cinnamon oil. With the increase in% concentration of cinnamaldehyde, there was increase in% inhibition of bacteria (*Providencia rettgeri).* Maximum antibacterial inhibition was 89.3% with 0.05% concentration of cinnamaldehyde. With the increase in% concentration of ciprofloxacin, there was increase in% inhibition of bacteria (*Providencia rettgeri).* Maximum antibacterial inhibition was 83.8% with 0.5% concentration of ciprofloxacin.

From the results it can be concluded that inhibition of bacteria (*P. rettgeri*) by test drug cinnamaldehyde and standard drug ciprofloxacin was found to be significant (p<0.01).Though Cinnamon oil too showed antimicrobial activity but at a very low concentrations, this may be due to immiscibility of cinnamon oil in nutrient broth.

Zhang *et al* in 2015 reported that Cinnamaldehyde damaged the integrity of the bacterial membrane, decreased the membrane potential and affected the metabolic activity, thus inhibiting bacterial growth. The antibacterial activity was evaluated against *Escherichia coli* and *Staplylococcus aureus.* Cinnamon oil is a volatile oil, volatile oils contain hydroxyl (-OH) group. Bacterial inhibition mainly occur due to this hydroxyl (-OH) group.

Major components of cinnamon oil viz. cinnamyl acetate, linalool, methyleugenol, possess antimicrobial activity by the sequential inhibition of a common pathway, inhibition of protective enzymes. Among all the components of cinnamon oil, linalool possess highest antimicrobial activity.

Ciprofloxacin is a class of fluoroquinolones antibiotic. They target DNA gyrase, topoisomerase IV. For many gram + ve bacteria topoisomerase is the primary target. For many grams –ve bacteria DNA gyrase is the primary target. These enzymes help in DNA unwinding. After DNA unwinding DNA replication occurs but ciprofloxacin inhibits these enzymes thus inhibit DNA unwinding thus DNA replication also inhibited resulting in inhibition of bacteria. Firstly the effect is bacteriostatic further it becomes bactericidal. Evaluation of TMA formed from choline in presence of *P. rettgeri* and cinnamaldehyde was done using HPTLC. At 4 µg / ml concentration the choline was not metabolised to TMA. At 4 µg / ml concentration, TMA formation was inhibited.

Thus at this concentration i.e. 4µg/ml cinnamaldehyde inhibited bacteria in the present study.

Cinnamon oil showed significant inhibition of *P. rettgeri* as choline was not metabolised to TMA. TMA formation was inhibited at concentration of 1µg/ml.

Further, HPTLC analysis showed that cinnamon oil is better than pure cinnamaldehyde because at the same concentration say at 1µg / ml cinnamon oil showed -154.24 concentration of TMA which means at this concentration i.e. 1µg/ml choline was not metabolized to form TMA. Hence, TMA formation

was inhibited. However, at same concentration of cinnamaldehyde i.e. at 1µg/ml cinnamaldehyde showed 115.65 concentration of TMA which means at this concentration choline was metabolised to form TMA. Thus TMA formation was not inhibited.

So, cinnamon oil is better in controlling of choline to TMA conversion in comparative to cinnamaldehyde. Because cinnamon oil contains other antimicrobial substances like linalool, this may results inhibition of bacterial growth. The present *in vivo* studies showed that choline diet (2%) when fed to C57BL/6J mice produced a significant increase ($p < 0.001$) in total cholesterol, mean triglyceride, LDL-cholesterol, VLDL-cholesterol levels, along with significant decrease ($p<0.001$) in mean HDL-cholesterol levels.

Cinnamaldehyde (35mg/mice/day) treated group i.e. group III (Preventive) and group IV (Curative) and cinnamon oil (46mg/mice/day) treated group i.e. group V along with choline diet showed significant decrease in total cholesterol levels ($p<0.001$), triglyceride levels ($p<0.01$) and significant increase ($p<0.001$) in HDL- cholesterol levels as compared to toxic control group i.e. group II which were better than the effects produced by the standard drug, ciprofloxacin(100mg/mice/day) in which there was no significant decrease ($p>0.05$) in total cholesterol, triglycerides, LDL-Cholesterol, VLDL-Cholesterol level and ARP indices as compared to toxic control group i.e. group II.

Results of the present study suggest that Cinnamaldehyde inhibited FMO3 enzyme thus, reducing the risk of atherosclerosis. Both cinnamaldehyde and cinnamon oil showed significant antimicrobial activity against *Providencia rettgeri*. Cinnamaldehyde and cinnamon oil alters the lipid profile in C57BL/6J mice. Both cinnamaldehyde and cinnamon oil acts as anti-hyperlipidemic agent From the results it can be concluded that test drug i.e. cinnamaldehyde showed significant anti-atherosclerotic potential in choline – fed C57BL/6J mice.

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