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Identification of the Antagonistic Effect of (-) Carvone On Ca^{2+} Channel, Histamine and Acetylcholine-Muscarinic Receptors in Isolated Smooth Muscle Preparation: An *In Vitro* and *In Silico* Correlation Study

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ABSTRACT

Carvone is a monoterpene found mainly in caraway seeds, dill and fennel fruits. These are used as a folk remedy for diarrhoea, acidity and other gastric disorders. The study was designed to evaluate the pharmacological effect of (-)Carvone mediated through L-Type voltage dependant Calcium [Ca^{++}] channel, Histamine [H_1] and Muscarinic [M_3] receptor on Isolated smooth muscle preparation by Dale's apparatus and its in –silico correlation. There are two phases in this study, in phase one, the antagonist effect of (-)Carvone on H_1, M_3 receptor and L-Type voltage gated Ca^{++}channel on isolated chicken ileum preparation by using Dale's apparatus. In phase two, to gain better insight for the interactions between the compounds and their respective targets along with (-) Carvone, we docked and validated its results based on scoring function using AUTODOCK 1.5.6. Here (-)Carvone showed ,the binding energy value of -7.53 Kcal/mol for Ca^{++} protein, -6.33 Kcal/mol for H_1 receptor, -5.82 Kcal/mol for M_3 receptor. It involves the active site residues and a crucial H-Bond interaction of key residues of LYS97, TYR431 and SER151 respectively. This results confirms that (-)Carvone docks well when compared to standard Ca^{++} blockers, anti-Histaminic drugs and anti-muscarinic drugs. The phase one study revealed that, the maximum inhibitory action was produced by (-)Carvone against Calcium ion, followed by acetylcholine and Histamine on isolated smooth muscle . The study shows, (-)Carvone produces antagonist effect by multi receptor mechanism against H_1, M_3 receptor and Ca^{++} so it may inhibit eosinophil, chemotaxis, LTB_4 release and various effects on many excitable cells of the body such as cardiac muscle, smooth muscles of blood vessels or neurons. The correlation of in –vitro and in-silico results suggested that (-)Carvone is useful for the treatment of various metabolic disorders and GI related disorders.

Keywords: (-)Carvone, Dale's apparatus, Calcium channel, Histamine, Muscarinic receptor, AUTODOCK 1.5.6.

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INTRODUCTION

The term receptor is used in pharmacology to denote a class of cellular macromolecules that are concerned specifically and directly with chemical signaling between and within cells. Combination of a hormone, neurotransmitter, or intracellular messenger with its receptor(s) results in a change in cellular activity. Hence, a receptor must not only recognize the particular molecules that activate it, but also, when recognition occurs, alter cell function by causing, for example, a change in membrane permeability or an alteration in gene transcription. With the concept of the receptor established, pharmacologists turned their attention to understanding the quantitative relationship between drug concentration and the response of a tissue. This entailed first, finding out how the fraction of binding sites occupied and activated by agonist molecules varies with agonist concentration and second, understanding the dependence of the magnitude of the observed response on the extent of receptor activation.

The majority of isolated compound show a remarkably high correlation of structure and specificity to produce pharmacological effects. Experimental evidence indicates that drugs interact with receptor sites localized in macromolecules. In most cases a rather specific chemical structure is required for the receptor site and a complementary drug structure. Slight changes in the molecular structure of the drug may drastically change specificity [1].

Organ isolation and tissue preparation

Isolated organs and tissue preparations have been extensively used for measuring effects due to receptor-agonist interactions, as well antagonist. The isolated ileum is probably the most widely used model in experimental pharmacology [2] Guinea pigs, rabbits and rats are usually the common sources of isolated tissues. Mice are also sometimes used for the purpose, while cats and dogs are too big to be sacrificed just for a piece of tissue [3]. The use of isolated preparations becomes an essential part in experimental pharmacology to understand the effects of a drug, especially in academics. The Committee for the Purpose of Control and Supervision of Experiments on Animals [CPCSEA] [4] is an authority which monitors animal experiments conducted in institutions through ethics committees and is mainly concerned with promoting the human care of animals used in biomedical and behavioral research. The use of an alternative source serves the purpose of decreasing, if not completely eliminating, the killing of laboratory animals just for a strip of tissue. The isolated strips of intestine are the most commonly used smooth muscle preparation, especially the ileum which possesses muscarinic, histaminic, adrenergic, serotonergic and GABAergic receptors. The presence of muscarinic receptors in goat ileum has been reported. It is both ethical and economical in using goat ileum for this purpose [5]. The purpose of bioassay is to ascertain the potency of a drug and hence it serves as a quantitative part of any screening procedure. Several methods are used to determine the potency of a drug substance with minimal error. The investigation sought to examine the suitability of the chicken ileum for doing pharmacological basic receptor finding. The study designed for pharmacological evidence of (-)Carvone mediated through L-Type voltage dependant Ca^{++}channel, $H_1$and M_3 receptor in isolated smooth muscle preparation and its in- silico correlation.

MATERIALS AND METHODS

Tissue selection

The drug was formulated into a suitable dosage form and used for biological study. The study needs to be carried out in-vitro and therefore no need of laboratory animals which has to be eventually sacrificed, for performing the in-vitro study. However, the chicken ileum is a tissue that is easily available from slaughter house and animal need not to be sacrificed for experimental purpose [6].

Chemicals & Drugs

(-)Carvone used in this experiment was from Sigma, St. Louis, USA. Histamine was purchased from Himedia Laboratories, Mumbai, India. Acetylcholine was purchased from Research Lab Fine Chemicals, Mumbai, India. Diltiazem was purchased from Torrent pharmaceuticals, Mehsana, India. Pheniramine was purchased from Aventis Pharma limited, Gujarat, India. Atropine was purchased from Titan Biotech, Bhiwadi,

India. Physiological salt solution compositions were purchased from NICE chemicals, Kerala, India. All the chemicals used in this experiment were of analytical grade.

***In-Vitro* Method**

In-vitro study was conducted using Dale's apparatus.

***In-Silico* Material**

AUTODOCK is a suite of automated docking tools. It is designed to predict how small molecules, such as substrates or drug candidates, bind to a receptor of known 3D structure. The structures of compounds were obtained from protein data bank and energy minimization was done using Argus lab, to obtain local minimum structure. Such energy minimized structures were considered for Docking and corresponding Pdbqt files were prepared in Auto-dock 1.5.6 [7].

Research Methodology

***In- Vitro* Methods**

Physiological salt solution composition

The superfusing Tyrode solution (pH 7.4; Temperature, 37± 1 ºC) was prepared using the following constituents in concentrations (in mM) KCl 2.68, NaCl 136.9, $MgCl_2$ 1.05, $NaHCO_3$ 11.90, NaH_2PO_4 0.42, $CaCl_2$ 1.8 and Glucose 5.55. The superfusion medium was filtered successively through filters, prior to use and was equilibrated with mixture of O_2: CO_2 (95: 5) at 37 ˚C for 1 hr before and throughout the superfusion.

Drug preparation

Preparation of Atropine

A stock solution of 1µg/ml was prepared using distilled water and was added to the inner organ bath and used as an antagonist of M_3 receptor.

Preparation of Diltiazem

A stock solution of 100µg/ml of Diltiazem was prepared and the following working concentrations were withdrawn from stock solution: 0.02µg, 0.04µg, 0.08µg and 0.12µg and was added to inner organ bath and used as an antagonist of Ca^{++} channel.

Preparation of Pheniramine

A stock solution of 100µg/ml of Pheniramine was prepared from which 1ml was withdrawn and made upto 100ml to get 1µg/ml stock solution [8]. The following working concentrations were withdrawn from stock solution: 0.2µg, 0.4µg, 0.8µg, 1.6µg, 3.2µg and 6.4µg and was added to inner organ bath and used as an antagonist of H_1 receptors.

Preparation of Histamine

A stock solution of 100µg/ml was prepared with distilled water. From the stock solution, various doses of the drug was withdrawn at concentration of 10µg, 20µg, 40µg and 80µg and was added to the inner organ bath and used as an agonist of H_1 receptor.

Preparation of Acetylcholine

A stock solution of 5µg/ml was prepared with distilled water. From the stock solution, various doses of the drug was withdrawn at concentrations of 0.5µg, 1µg, 2µg, 4µg and 8µg and added to the inner organ bath and used as an agonist of M_3 receptor.

Preparation of Calcium chloride

A stock solution of 2% $CaCl_2$ was prepared using distilled water. From the stock solution, various doses of drug were withdrawn at concentrations of 1mg, 2mg, 4mg, 8mg, 16mg and 32mg and added to the inner organ bath and used as a Ca^{++} channel agonist.

Preparation of Barium chloride

A stock solution of 0.5% $BaCl_2$ was prepared using distilled water. From the stock solution, various doses of drug were withdrawn at concentrations of 0.5mg,1mg, 2mg, 4mg, 8mg and 16mg and was added to the inner organ bath and used as a Ca^{++} channel stimulant.

Preparation of (-)Carvone

The solubility of (-) Carvone was established using Tween 80. Tween 80 was employed because (-) Carvone being a volatile oil was found to be insoluble in water, 10% alcohol and DMSO. A homogenous stock solution of 30ml containing 100 µl of drug was prepared using Tween 80 (20µl). From this stock solution the working concentration of drug doses was prepared. (-) Carvone was used as the test solution throughout the experiment.

Chicken ileum preparation and procedure of dose response curve recording

Fresh chicken ileum was obtained from a local slaughter house in 250ml of warm tyrode solution and transported to laboratory where it was aerated. The ileum was cleared off its intestinal contents and mesenteric attachments. A small portion of 2-3cm of ileum was used and carefully removed the fatty material while ensuring proper aeration. The ileum was suspended in the organ bath containing 30ml of Tyrode solution and gassed with 95% O_2 / 5% CO_2 [9]**.** The tissue was kept for a stabilization period of one hour while the temperature was maintained at 37 ± 1ºC. "3-6 fold magnification was adjusted with the resting load of 1g". The tissue was washed at an interval of 10 minutes regularly up to 1hour. DRC (Dose Response Curve) was recorded using the standard Histamine, Calcium chloride, Acetylcholine, Barium chloride solution and their antagonist effect was also recorded by using (-)Carvone as well as with standard drug in dose dependent manner. Effects of drugs were recorded on smoked kymograph by means of an isotonic frontal writing lever. The baseline was noted for 30 seconds and the contact time for drug action was 60 seconds. The tissue was perfused three times at an interval of 1 minute by using physiological salt solution between every dose added.

Comparative analysis of IC_{50}

The dose Vs percentage response graphs of antagonists used in the study were plotted, from which IC_{50} values were compiled using Microsoft Excel. It was presented in graphical format using bar diagram where IC_{50} values of antagonist were compared individually to IC_{50} value of (-)Carvone.

Determination of pA_2 and pD'_2 values

The determination of pA_2 values of competitive antagonist-Diltiazem, Pheniramine and Atropine and pD'_2 values of non-competitive antagonist-(-)-carvone using IC_{50} values. From this study their relative potencies were determined using respective formulas.

pA_2= -log (Molar concentration of IC_{50} of competitive antagonist)
pD'_2= -log (Molar concentration of IC_{50} of non-competitive antagonist)

In-silico Methods

Molecular Docking

The molecular level interaction between compounds mentioned above and the receptors 3RZE (Histamine H_1), 4DAJ (M_3 receptors), 3UON (M_2 receptors), 3VYV (Calcium channel) were carried out to gain better understanding. AUTODOCK 1.5.6 was employed in the study to dock the compounds to the receptors.

Autodock requires the receptor and ligand co-ordinates in either Mol2 or PDB format. The water molecules, ligand, sulphates [SO_4], phosphates [PO_4], solvents, salts etc were deleted so as to obtain pure receptor structure for docking. Polar hydrogen atoms were added to the receptor file and their partial charges were added to the corresponding carbon atoms. The receptor PDB file was transformed into the PDBQT format file containing the receptor atom co-ordinates, partial charges and solvation parameters. The ligand file was transformed into a PDBQT file. Merged non-polar hydrogen atom and torsions are defined. The grid calculations were setup and maps were calculated with the program Auto Grid. The grid maps were centered on ligand binding site and were of dimension 60×60×60 points. The grid spacing was 0.375 Å and the default Auto-dock parameter setting were used for docking. All docking runs were performed thrice using the Lamarckian genetic algorithm and the obtained dock scores were reported in Kcal/mol**.** The docking protocol utilized in the study consisted of 25 independent runs per ligand, using an initial population of 50 randomly placed individuals, a maximum number of 2.5× 105 energy evaluations, a mutation rate of 0.02, cross over rate of 0.80, and an elitism value of 1. The probability of performing a local search on an individual in population was 0.06, using a maximum of 300 iterations [11]. From the above literature review we took the β subunits as our target of study and docked the Monoterpene and validated its results based on scoring function using AUTODOCK 1.5.6.

RESULTS

Results of *in-vitro* Method

Antagonistic effect of standard drugs on smooth muscle

Antagonist effect of Diltiazem against Calcium Chloride on isolated smooth muscle

The agonist effect of 0.5 % of Calcium chloride at the dose of 0.1, 0.2, 0.4, 0.8 and 1.6ml caused a concentration- dependent muscle contraction. The lower concentration of calcium chloride (0.1ml of 0.5%) produced the minimal contraction of myocyte. The maximum depolarization caused by calcium chloride was at the dose of 1.6ml of 0.5 % Calcium chloride. It is expressed as ceiling effect. Diltiazem at the dose of 0.04mg reduced to nearly half the response produced by 0.8ml of 0.5% Calcium chloride. This value may be considered as IC_{50} value. Diltiazem at the concentration of 0.12 mg completely antagonized the effect of calcium chloride in smooth muscle. The agonist and antagonist effect of calcium chloride and Diltiazem on smooth muscle preparation is presented in **Table1** and **Figure 1.**

Table 1: Agonist effect of $CaCl_2$ and antagonist effect of Diltiazem on smooth muscle

Agonist action of $CaCl_2$		Antagonist action of Diltiazem (Diltiazem VS $CaCl_2$)			
0.5% $CaCl_2$ (ml)	Response (mm)	Diltiazem (mg)	0.5% $CaCl_2$ (ml)	Response (mm)	% Inhibition
0.1	4	0	0.8	9	0.00
0.2	8	0.02	0.8	7	22.22
0.4	6	0.04	0.8	4	55.55
0.8	9	0.08	0.8	1	88.88
1.6	9	0.12	0.8	0	100

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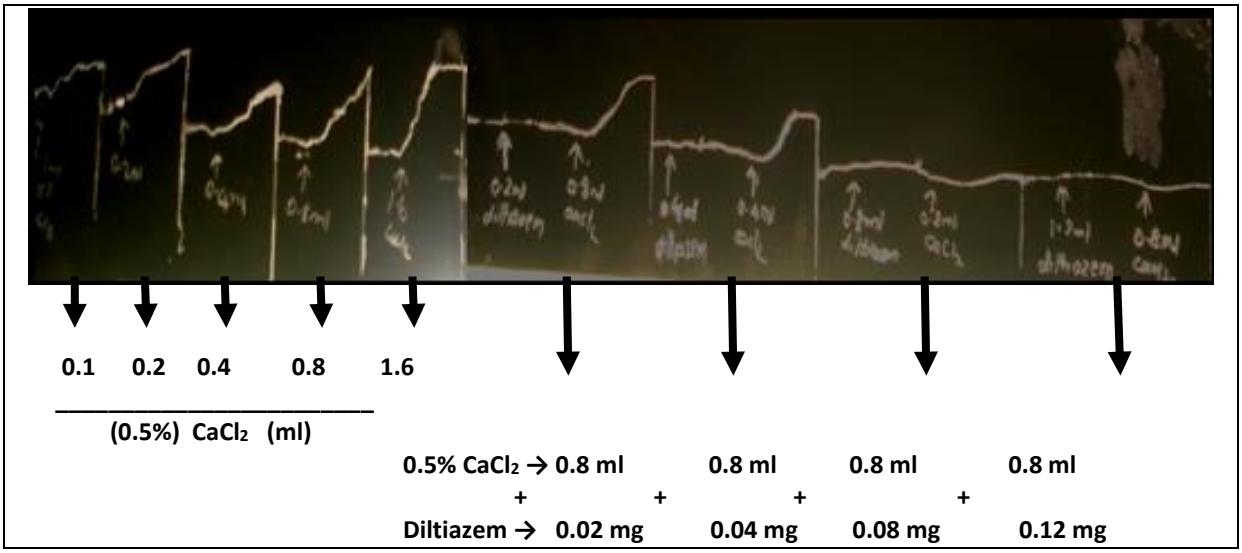
Figure 1: DRC of $CaCl_2$ and effect of Diltiazem against $CaCl_2$ on isolated smooth muscle



Antagonistic effect of Pheniramine against Histamine on isolated smooth muscle

The antagonistic effect of Pheniramine against histamine on isolated smooth muscle strip was recorded. The muscle spasm was produced when histamine was added into bath fluid at the concentration of 10µg to 160µg. Reference doses from 0.2µg to 6.4µg of Pheniramine showed antagonistic action. Pheniramine in the concentration of 3.2 µg and 6.4µg markedly antagonized the effect of histamine. Pheniramine at the concentration of 0.2µg reduced contractile response of histamine on isolated smooth muscle to 50% (IC_{50}). The antagonist effect of Pheniramine is presented in **Table 2** and **Figure 2.**

Table 2: Agonist effect of histamine and antagonist effect of Pheniramine on smooth muscle

Agonist action of Histamine		Antagonist action of Pheniramine (Pheniramine VS Histamine)			
Histamine (µg)	**Response (mm)**	**Pheniramine (µg)**	**Histamine (µg)**	**Response (mm)**	**% Inhibition**
10	8	00	80	22	0.00
20	10	0.2	80	11	50.00
40	18	0.4	80	10	54.50
80	22	0.8	80	10	54.50
160	13	1.6	80	10	54.50
-	-	3.2	80	08	63.63
-	-	6.4	80	05	77.27

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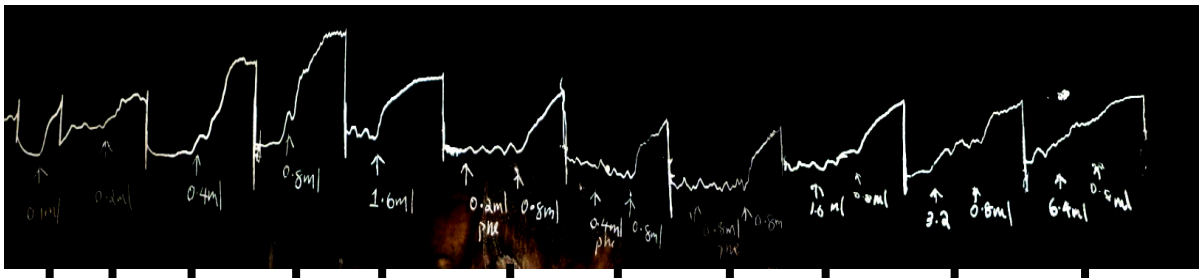
Figure 2 :DRC of Histamine and effect of Pheniramine against Histamine on isolated smooth muscle



10 20 40 80 160

Histamine (µg)

P (µg) → 0.2 + 0.4 + 0.8 + 1.6 + 3.2 + 6.4

H (µg) → 80 + 80 + 80 + 80 + 80 + 80

P (µg) = Pheniramine (µg)

H (µg) = Histamine (µg)

Antagonist action of Atropine against Acetylcholine on isolated smooth muscle

Acetylcholine at the concentration of 0.5 µg, 1µg, 2µg, and 4µg caused a concentration-dependent muscle depolarization that was associated with an increase in muscle contraction. The lower concentration of acetylcholine (0.5µg) produced the initial contraction of muscle. The maximum depolarization was caused by acetylcholine at the concentration of 4µg. It is expressed as ceiling effect. The effect of Atropine (1µg) was antagonized by increasing doses of acetylcholine (0.5µg -4.0µg) thereby suggesting the competitive nature of interaction between these two drugs. The agonist and antagonist effect of Acetylcholine and Atropine on smooth muscle preparation is presented in **Table 3 and Figure 3.**

Table 3: Agonist effect of acetylcholine and antagonist effect of Atropine on smooth muscle

Agonist action of Acetylcholine		Antagonist action of Atropine (Acetylcholine VS Atropine)			
Acetylcholine (µg)	Response (mm)	Atropine (µg)	Acetylcholine (µg)	Response (mm)	% Inhibition
0.5	12	0.0	0.0	00	00.00
1.0	21	1.0	0.5	11	62.06
2.0	26	1.0	1.0	11	62.06
4.0	29	1.0	2.0	13	55.17
8.0	29	1.0	4.0	14	51.72

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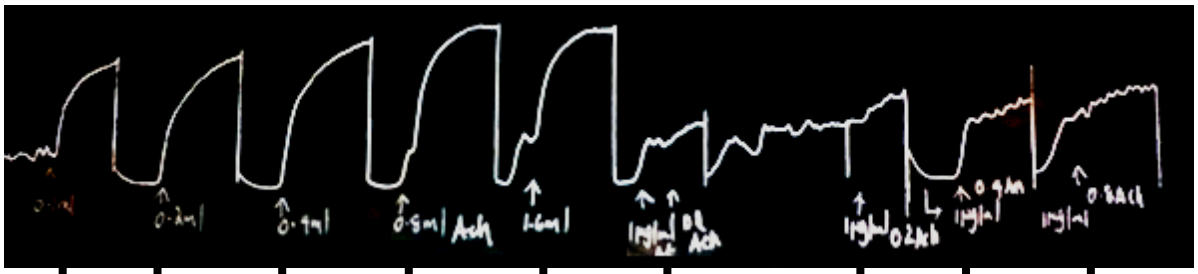
Figure 3: DRC of acetylcholine and effect of atropine against acetylcholine on isolated smooth muscle



0.5 1.0 2.0 4.0 8.0

Ach (µg)

AT (µg) →	1.0	1.0	1.0	1.0
+	+	+	+	
Ach (µg) →	0.5	1.0	2.0	4.0

AT (µg) = Atropine (µg)
Ach (µg) = Acetylcholine (µg)

III. Antagonistic effect of (–)-carvone against standard drugs

Effect of (-)-carvone against $CaCl_2$ on isolated smooth muscle

The effect of (–)-carvone against voltage gated calcium channel was studied. 2% $CaCl_2$ (0.05ml to 1.6ml) produced smooth muscle contraction. The effect of different concentrations of (–)-carvone (1.5840µg, 2.3712µg and 3.9552µg) was studied against a fixed concentration of $CaCl_2$ (0.8ml of 2% solution) .At concentration of 1.5840µg (-)-carvone showed 50% inhibition of the response. At concentration of 3.9552µg (–)-carvone showed 100% inhibition of the response. The antagonist effect of (–)-carvone is presented in **Table 4** and **Figure 4.**

Table 4: Agonist effect of $CaCl_2$ and antagonist effect of (–)-carvone on smooth muscle

Agonist action of $CaCl_2$		Antagonist action of (–)-carvone ((–)-carvone VS $CaCl_2$)			
2% $CaCl_2$ (ml)	Response (mm)	(–)-carvone (µg)	2% $CaCl_2$ (ml)	Response (mm)	% Inhibition
0.05	6	0.0000	0.0	0	-
0.1	14	0.0000	0.0	0	-
0.2	19	0.0000	0.0	0	-
0.4	22	1.5840	0.8	14	50.00
0.8	28	2.3712	0.8	5	82.14
1.6	22	3.9552	0.8	0	100.00

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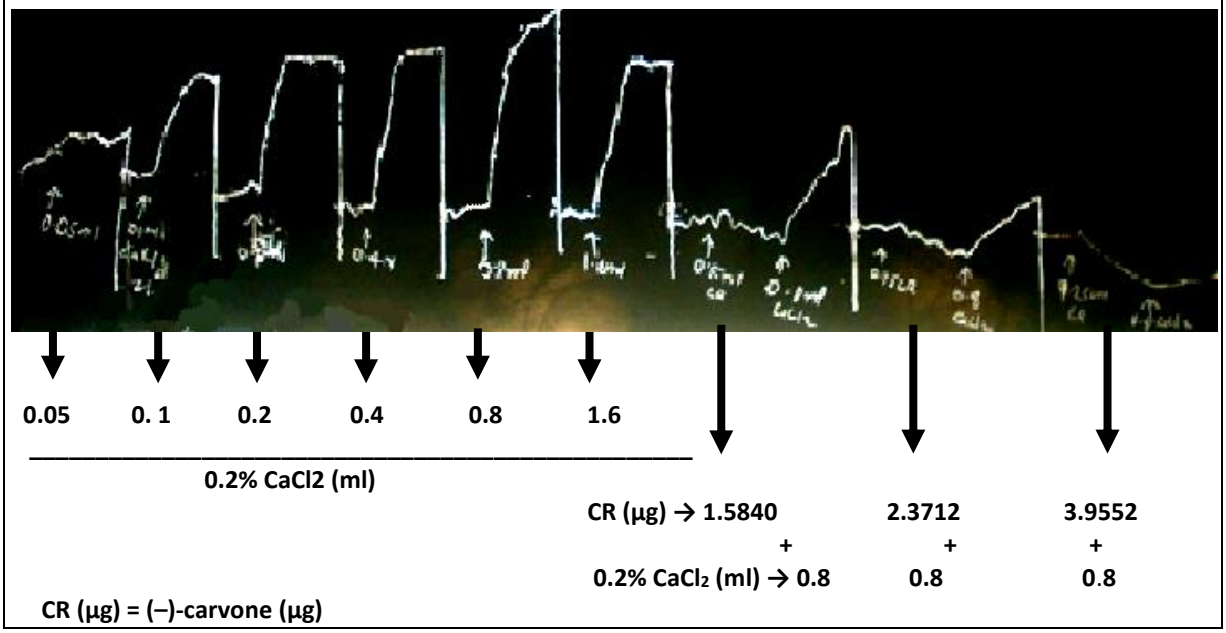
Figure 4: DRC of $CaCl_2$ and effect of (–)-carvone against $CaCl_2$ on isolated smooth muscle



Effect of (-)-carvone against histamine on isolated smooth muscle

The effect of (–)-carvone against Histamine 1 receptor was studied. The smooth muscle contraction was stimulated by histamine dose from 10µg to 80µg. The effect of different concentration of (–)-carvone (1.5840µg, 2.2176µg, 2.8512µg and 3.4848µg) was studied using a fixed dose of histamine at concentration of 80µg. (–)-carvone at the concentration of 1.5840µg showed 50% inhibition of response against histamine. Moreover, the (–)-carvone at concentration of 3.4848µg showed 100% inhibition against reference dose of histamine (80 µg). The antagonist effect of (–)-carvone is presented in **Table 5** and **Figure 5.**

Table 5: Agonist effect of Histamine and antagonist effect of (–)-carvone on smooth muscle

Agonist action of Histamine		Antagonist action of (–)-carvone ((–)-carvone VS Histamine)			
Histamine (µg)	Response (mm)	(–)-carvone (µg)	Histamine (µg)	Response (mm)	% Inhibition
10	13.0	1.5840	80	7	53.33
20	14.0	2.2176	80	3	80.00
40	14.5	2.8512	80	1	93.33
80	15.0	3.4848	80	0	100.00

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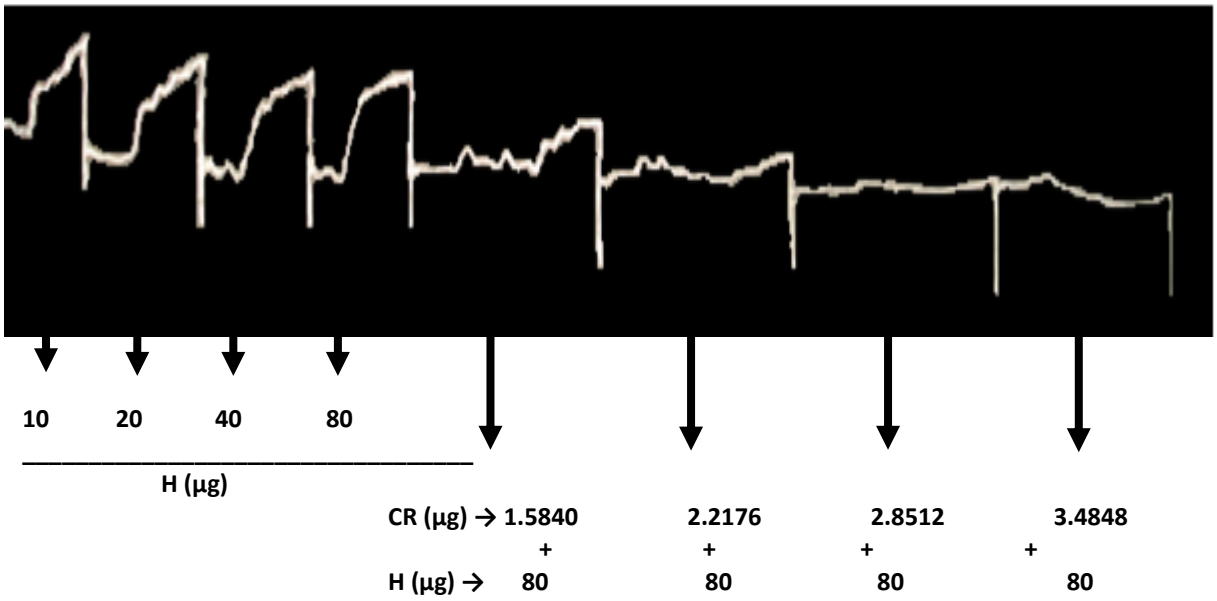
Figure 5: DRC of histamine and effect of (–)-carvone against histamine on isolated smooth muscle



CR (µg) = (–)-carvone (µg)
H (µg) = Histamine (µg)

The effect of (-)-carvone against Acetylcholine on isolated smooth muscle

The effect of (–)-carvone against Acetylcholine, type 3 receptor was studied. The smooth muscle contraction was depolarized by acetylcholine dose from 0.5µg to 8µg.The effect of different concentrations of(–)-carvone(1.2672µg, 1.5840µg, 2.8512µg, 1.9008µg, 2.5344µg, 3.1680µg, and 6.3360µg) was studied against a fixed standard dose of 4µg Acetylcholine.(–)-carvone at the concentration of 1.5840µg showed 50% inhibition of response against histamine. (–)-carvone showed 100% inhibition of response at the dose of 6.3360µg against reference dose of acetylcholine(4µg) and it is presented in **Table 6** and **Figure 6.**

Table 6: Agonist effect of Acetylcholine and antagonist effect of (–)-carvone on smooth muscle

Agonist action of Acetylcholine		Antagonist action of (–)-carvone ((–)-carvone VS Acetylcholine)			
Acetylcholine (µg)	Response (mm)	(–)-carvone (µg)	Acetylcholine (µg)	Response (mm)	% Inhibition
0.0	00.0	1.2672	4	20	37.50
0.5	17.5	1.5840	4	16	50.00
1.0	26.0	1.9008	4	13	59.37
2.0	30.0	2.5344	4	11	65.62
4.0	32.0	3.1680	4	8	75.00
8.0	33.0	6.3360	4	0	100

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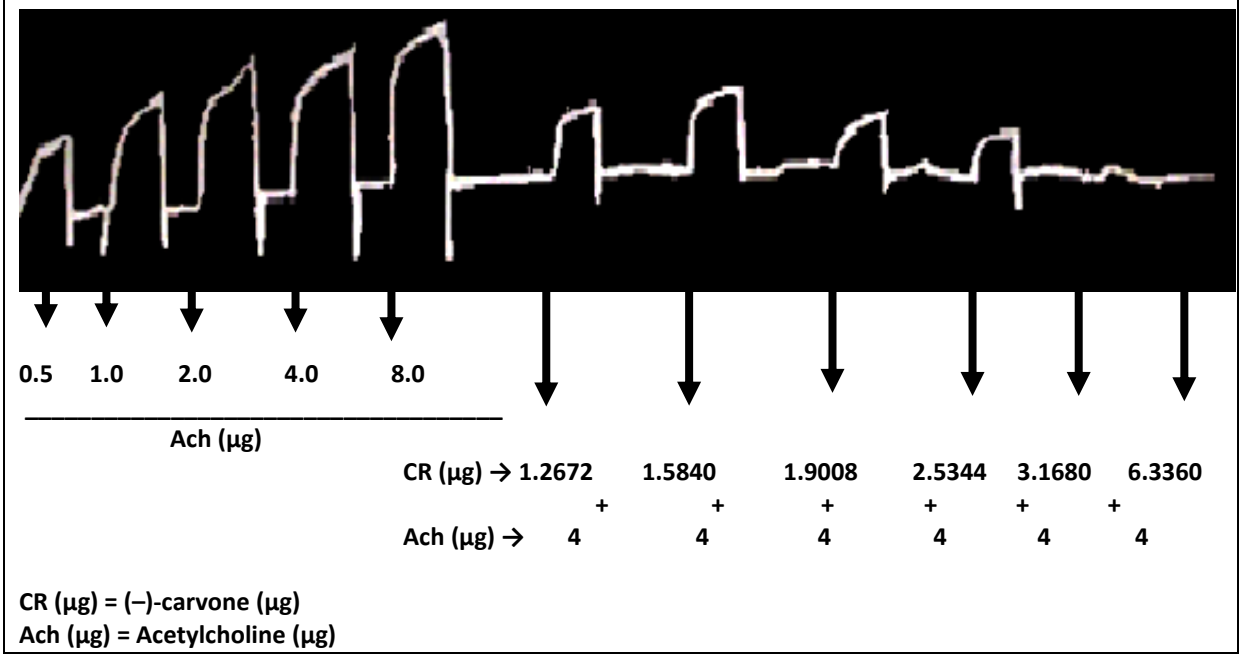
Figure 6: DRC of Acetylcholine and effect of (–)-carvone against Acetylcholine on isolated smooth muscle



CR (µg) = (–)-carvone (µg)
Ach (µg) = Acetylcholine (µg)

Complete antagonist effect of (-)-carvone against $BaCl_2$ on isolated smooth muscle

The effect of (–)-carvone against channel based mechanism was studied. 0.5% $BaCl_2$ (0.05ml to 1.6ml) produced smooth muscle contraction. The effect of different concentrations of (–)-carvone (0.7920µg, 1.5840µg and 2.3712µg) was studied against a fixed concentration of $BaCl_2$ (0.8ml of 0.5% solution) .At concentration of 0.7920µg (-)-carvone showed almost 50% inhibition of the response. (-)-carvone at a concentration of 2.3712 showed 100% inhibition of response against fixed dose of barium chloride (0.8ml of 0.5% solution). The antagonistic effect (-)-carvone is presented in **Table 7** and **Figure 7.**

Table 7: Agonist effect of Barium chloride and antagonist effect of (–)-carvone on smooth muscle

Agonist action of $BaCl_2$		Antagonist action of (–)-carvone ((–)-carvone VS $BaCl_2$)			
0.5% $BaCl_2$ (ml)	Response (mm)	(–)-carvone (µg)	0.5% $BaCl_2$ (ml)	Response (mm)	% Inhibition
0.05	8.0	0.0000	0.0	0	00.00
0.1	9.0	0.0000	0.0	0	00.00
0.2	10.0	0.0000	0.0	0	00.00
0.4	11.3	0.7920	0.8	9	48.57
0.8	17.5	1.5840	0.8	2	88.57
1.6	18.0	2.3712	0.8	0	100

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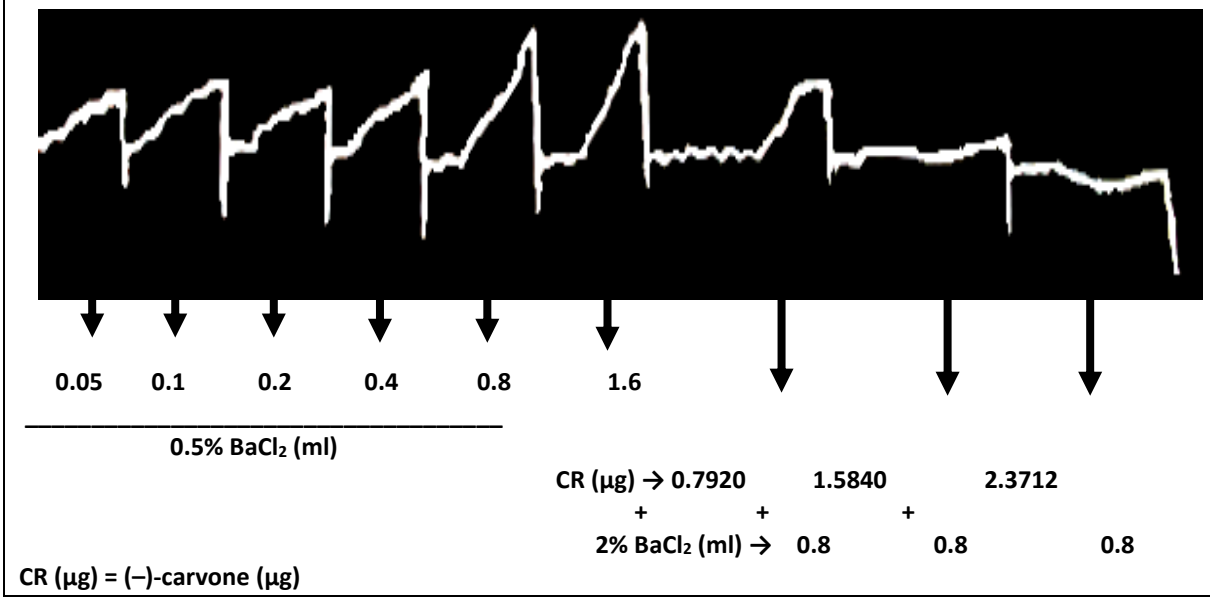
Figure 7: DRC of $BaCl_2$ and effect of (–)-carvone against $BaCl_2$ on isolated smooth muscle



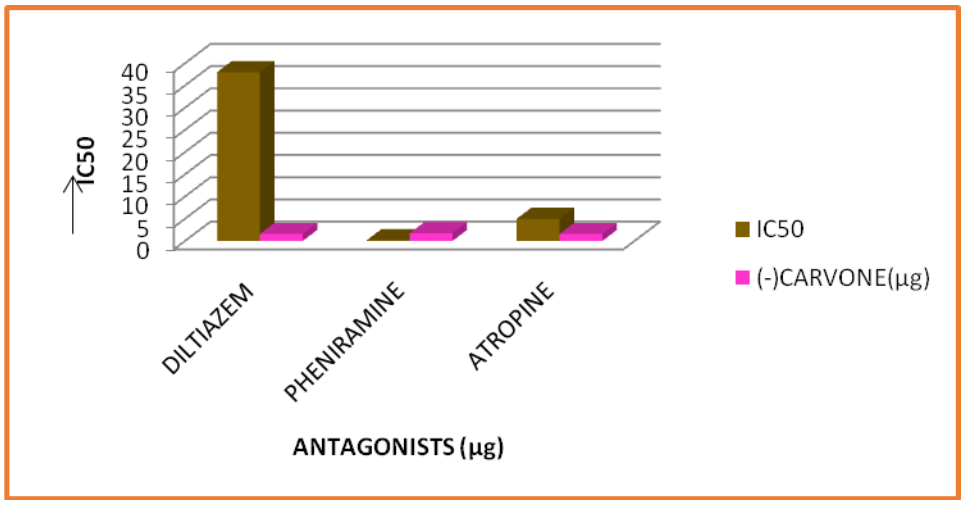
Comparative analysis of IC_{50}

The dose versus percentage response graphs of antagonists used in the study were plotted, from which IC_{50} values were compiled using MICROSOFT EXCEL. It was presented in graphical format using bar diagram where IC_{50} values of antagonist were compared individually to IC_{50} value of (-)-carvone. Results are shown in **Table 8** and **Figure 8.**

Table 8: IC_{50} values of (-) carvone and standard antagonists

Agonist	IC_{50} of (-) Carvone(µg)	Antagonist	IC_{50}
Calcium chloride	1.584	Diltiazem	38
Histamine	1.760	Pheniramine	0.2
Acetylcholine	1.584	Atropine	5

Figure 8: Comparative study of ic_{50} of standard antagonist with (-)carvone



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Determination of pA_2 and pD'_2 values

The determination of pA_2 values of competitive antagonist-Diltiazem, Pheniramine and Atropine and pD'_2 values of non-competitive antagonist-(-)-carvone using IC_{50} values. From this study their relative potencies were determined using respective formulas and are shown in **Table 9**.

pA_2= -log (Molar concentration of IC_{50} of competitive antagonists)
pD'_2= -log (Molar concentration of IC_{50} of non-competitive antagonist

Table 9: Analysis of relative potencies of antagonists

Agonist	pD'_2 of (-) carvone	Antagonist	pA_2
Calcium chloride	5	Diltiazem	4.09
Histamine	5	Pheniramine	6.096
Acetylcholine	5	Atropine	4.769

Results of *in-silico* Method

Calcium channel blockers:

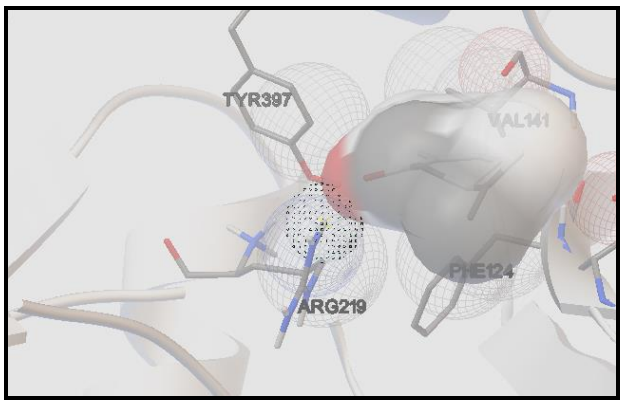
We took some of the common calcium channel blockers that are currently available and performed blind docking which involved the Chain A of the β-subunit of calcium channel structure obtained from the Protein Data Bank (PDB). So we docked all the compounds and results of standard drugs and (-)-carvone were obtained and are tabulated in **Table 10** and result in **Figure 9**

This results suggests that (-)-carvone with an acceptable binding energy of -7.53 Kcal/mol has similar potency like the available standard Ca^{2+} channel blockers.

Table 10: Docking score of Ca^{2+} channel antagonists

Sl. No:	Drug	Binding Energy (kcal/mol)
1	Nifedipine	-6.62
2	Nisoldipine	-7.63
3	Diltiazem	-8.32
4	**(-) Carvone**	**-7.53**

Figure 9: Conformational orientation of (-) Carvone with H-bond interactions



Docking results of (-)-carvone:

The molecular docking results of (-) Carvone showed the binding energy value of -7.53 Kcal/mol. It involves the active site of chain A of calcium channel and a crucial H-bond interaction of LYS97: HZ21.

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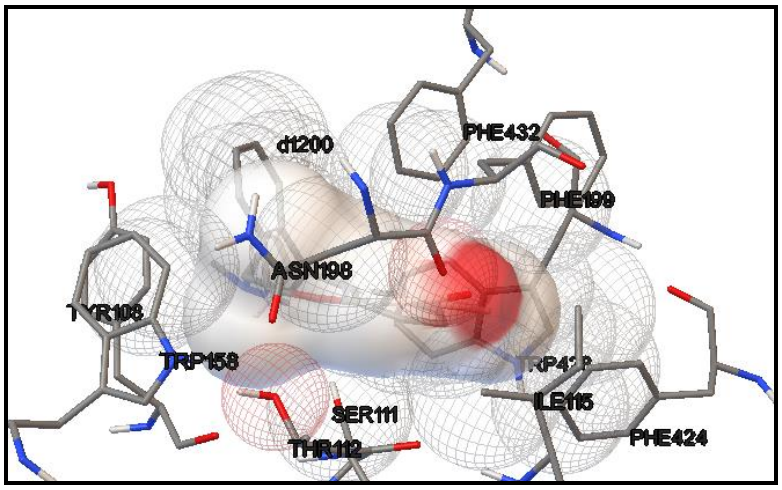
Histamine receptor (H_1) antagonism:

In this study commonly used H_1 antagonists and docked all of them with the Histamine-1 receptor obtained from PDB and results of standard drugs and (-) Carvone were obtained and are tabulated in **TABLE 11** and result in **FIGURE 10**

Table 11: Docking Score Of H_1 Antagonists

S.No:	Drug	Binding Energy(Kcal/mol)
1	Diphenhydramine	-8.34
2	Pheniramine	-7.99
3	**(-)-carvone**	**-6.33**

Figure 10: Conformational orientation of (-)-carvone with H_1 receptor



(-)-carvone docked against the same target in the active site with some beneficial interactions with PHE 432A, THR112A, ASN198 without any H-bonding. The binding energy was found to be -6.33Kcal/mol, which is an acceptable one.

Muscarinic receptor (M_3) antagonism:

Here, we took the Anti-Cholinergics (Antispasmodics) that are currently available and performed blind docking which involved the Chain A of the M_3 receptor structure which was obtained from the PDB. So we docked all the compounds and results of standard drugs and (-)-carvone were obtained and are tabulated in **Table 12** and result in **Figure 11**

Table 12: Docking score of M_3 Antagonists

S.No:	Drug	Binding Energy(Kcal/mol)
1	Glycopyrrolate	-9.96
2	Dicyclomine	-8.77
3	Atropine	-8.58
4	**(-)-carvone**	**-5.82**

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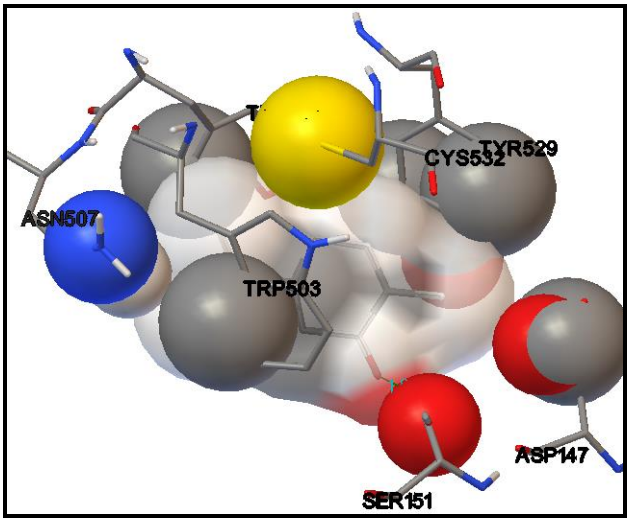
Figure 11: Conformational orientation of (-)-carvone with M_3



Conformational orientation of (-)-carvone with H-bond with SER151: HG

The molecular docking results of (-)-carvone showed the binding energy value of -5.82 Kcal/mol. It involves the H-bond interaction with SER151 residue. These results show that (-)-carvone is a mild M_3 blocker; it docks well into the active site of the Chain A of the M_3 receptor with a H-bond.

DISCUSSION

The purpose of this study was to evaluate the mechanism of action of (-)-carvone on targeted site. The correlation between in-vitro and in-silico findings are very supportive.

In in- vitro method, we targeted the action of (-)-carvone on the L- type voltage gated ion channel, H_1 receptor as well as M_3 receptor. It is recognized from the studies of Catterall WA [10] that voltage- gated Ca^{2+} channels are important for regulating entry of extracellular Ca^{2+} in smooth muscle cells. The influx of Ca^{2+} is essential for the development of muscle tension, which mediates arterial vasoconstriction, and ultimately influences systemic blood pressure. Karaki H et al [11] has demonstrated the contractile effects of the smooth muscles of the intestines are due to the cytosolic free calcium level. There is an exchange of calcium between extracellular and intracellular calcium stores. The voltage dependent calcium channels (VDCs) are responsible for the influx of calcium into the sarcoplamic reticulum [12, 13, 14]**.** This leads to periodic depolarization and repolarization of the intestinal tissues that accounts for its spontaneous responses. The effect of different concentration of calcium chloride on L-type calcium channel on smooth muscle strip has been demonstrated by Ghayur MN et al. Calcium chloride treated isolated tissues that open the voltage–operated calcium channels and allows the extra-cellular calcium into the cytosol resulting in the depolarization of tissues [15]. The present study confirmed that (-)-carvone, a selective L-type Ca^{2+} channel antagonist, elicits marked relaxation of intestinal smooth muscle. It completely antagonizes the effect against increased level of intracellular calcium ion. These findings indicate that the spasmolytic effect of (-)-carvone may be mediated through calcium channel antagonistic activity. It caused dose dependent relaxation of smooth muscle against intracellular calcium ion. Fleckenstein A had postulated that Calcium channel blockers such as verapamil, diltiazem, nifedipine interfere with smooth muscle contraction by binding to voltage sensitive calcium channels in a way that prevents depolarization induced Ca^{2+} influx [16]**.** Here we used diltiazem as a standard drug for comparison with (-)-carvone. (-)-carvone also produced similar effect as diltiazem against calcium overloading in intracellular level.

The property of depolarization produced by Ach on isolated smooth muscle from the chicken gastro intestinal tract was obtained. The interpretation of Ach results indicated could stimulate the mAch Receptor and induced depolarization. The muscarinic receptors mediating contraction of guinea-pig ileum (and indeed of many other smooth muscle preparations) are defined pharmacologically as M_3 by Eglen RM et al [17]. There is a large population of M_2 receptors in many smooth muscles, and it seems likely that they are involved in

antagonizing the relaxant effects of agents that elevate cAMP [18,19]. Another contrasting study by Bolton TB et al [20] states that M_2 receptors in guinea-pig ileum also stimulate the opening of cation-selective channels that depolarize the muscle cells.

Several studies have indicated that the receptor mediating relaxation of vascular smooth muscle (via release of relaxing factors from endothelial cells) is M_3 [21 22,23]. In our finding, (-)-carvone has moderate action in the pathway of mAch 3 receptor. It hence inhibits the action of Acetylcholine on smooth muscle.

H_1 receptors are distributed in a wide variety of tissues such as central nerves system, smooth muscles, gastrointestinal tract, cardiovascular system including endothelial cells and lymphocytes. Upon activation of the receptor stimulation of phospholipase C (PLC) pathway (and Phospholipases A_2 and D) via $G\alpha_q/_{11}$ leads to an increase of inositol 1,4,5- trisphosphate (IP_3) and 1,2- diacylglycerol (DAG) and thereby via increased Ca^{2+} concentration and /or cAMP, cGMP, NO formation to contraction of smooth muscles. H_1 receptor agonists are mainly used as pharmacological tools instead of therapeutically active drugs. In the present study, Histamine is used for stimulating Histamine H_1 receptor for smooth muscle contraction. Our observation in this study is that Histamine increases the contractile effects of intestinal smooth muscle [24]. (-)-carvone completely blocks the action of Histamine. (-)-carvone mechanism may be due to blocking the Histamine H_1 receptor on intestinal smooth muscle.

In interlink between histamine receptor and calcium channels are important for the study. Histamine binding to H_1 receptors on smooth muscle myocytes causes a dose dependent release of internal calcium that transiently raises cytosolic calcium concentration. The antagonist effect of (-)-carvone may block H_1 receptor indicate that it is due to H_1 receptor-coupled release of internal calcium stores.

Kotlikoff MI et al [25] conducted a study which illustrates that the Histamine-induced calcium release is mediated by inositol polyphosphate release that is mediated by Inositol polyphosphate synthesis. Histamine can trigger this synthesis in smooth muscle. Tracheal smooth muscle generates Inositol phosphates in response to Histamine and other agonists and IP_3 causes calcium release from intracellular stores of similar magnitude and time course in smooth muscle.

Our results demonstrated that (-)-carvone may also inhibit the histamine- induced calcium release in smooth muscle myocytes. Antagonist effect of (-)-carvone against histamine-induced calcium release by calcium ion suggests that protein kinase C activation modulates calcium release and may play an important feedback role in the regulation of phasic contraction in smooth muscle myocytes.

Our results in in- vitro discovery, highlight that (-)-carvone as an important inhibitor of calcium influx into the myocytes. To summarize, (-)-carvone exhibits spasmolytic activities mediated through L-Type voltage gated ion channel antagonist, mAch receptor antagonist and Histamine 1 receptor antagonist.

The effect of (-)-carvone on smooth muscle was assessed, finding that this compound non-competitively blocked Ach, Ca^{2+} and Histamine, all with the same pD'_2 values (about 5). It is defined as the -log [antagonist] (in Molar) that reduces to the half the effect of the agonist. This parameter estimates the affinity of the pure substance for its own receptor site, which differs from the agonist receptor. Also, the comparative analysis of IC_{50} of standard antagonists (Atropine, Diltiazem and Pheniramine) and their respective pA_2 values confirmed that (-)-carvone is not a competitive antagonist of Ach, Ca^{2+} and Histamine. Our results are hence comparable with study done by Consolini AE et al [26].

In-silico Studies showed that β - subunit of calcium channel marks the main action related to L-type voltage gated calcium channel. When analyzed, the compound (-)-carvone which docked well (-7.53Kcal/mol) in comparison with other standard drugs Nifedipine, Nisoldipine and Diltiazem (-6.62Kcal/mol, -7.63Kcal/mol and -8.32Kcal/mol) respectively. This suggested that, (-)-carvone has potent effect similar to standard calcium channel blockers. Similarly, (-)-carvone also docked well with an acceptable binding energy of -6.33 Kcal/mol on Histamine(H_1) receptor in comparison with other H_1 antagonists like Pheniramine and Diphenhydramine (-7.99Kcal/mol and -8.34Kcal/mol) respectively. Whereas (-)-carvone when docked on chain A of the Muscarinic(M_3) receptors showed mild affinity with a binding energy of -5.82Kcal/mol in comparison with standard antimuscarinic-antispasmodic drugs like Atropine, Dicyclomine and Glycopyrrolate (-8.58Kcal/mol,-8.77 Kcal/mol,-9.96Kcal/mol) repectively.

CONCLUSION

In this study, (-)-carvone binds effectively to the active site of target protein. The binding modes exhibited by various standard drug compounds illustrate the importance of specific residue H-Bonds within the active site region of calcium channel protein. From the study it is clear that the efficiency of binding of (-)-carvone within the active site of Ca^{2+} channel protein would get enhanced when H-Bonds are favored to the LYS97. These results clearly indicate that the use of (-)-carvone as a potential new lead compound against calcium channel antagonistic activity and it is comparatively better than standard drugs available in the market.

Present study indicate that, (–)-carvone is an antagonist, its effect include manifold pharmacological pathways such as histamine receptor, Acetylcholine blocking effect and calcium channel stimulating neurotransmitter inhibition on L- Type Calcium ion channel. The effect of (-)-carvone on smooth muscle was also assessed, finding that this compound non-competitively blocked Ach, Ca^{2+} and Histamine, all with the same pD'_2 of about 5. The correlation of in-vitro and in-silico results suggested that (–)-carvone is useful for treat various leading illness. However, it has potential blocking action against influx of calcium into myocytes. It may effect on many excitable cells of the body, such as cardiac muscle, vascular and non vascular smooth muscles, or neurons.Thus (-)-carvone can be utilized in various cardiovascular, neurological and other disorders like gastrointestinal tract, respiratory tract etc.

REFERENCES

[1] Foreman JC, Johansen T, Gibb AJ, editors. Textbook of receptor pharmacology. CRC press; 2010 Sep 10.
[2] Vogel HG, editor. Drug discovery and evaluation: pharmacological assays. Springer Science & Business Media; 2002 Jun 13.
[3] Ghosh MN. Some common evaluation technics chapter 25. Fundamentals of Experimental Pharmacology, Second edition, Scientific Book Agency, Calcutta, 1984; pp.144-152.
[4] CPCSEA Guidelines for laboratory animal facility; Chennai, CPCSEA: 2 -3
[5] Kulkarni S K. Hand book of Experimental Pharmacology, 3rd edition; Vallabh Prakashan; 2004.
[6] Sareesh NN, Rao JP. Phosphate transport by the everted intestinal and gallbladder sacs of the frog. Indian journal of physiology and pharmacology. 2006 Dec;51(3):301-2.
[7] Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK, Olson AJ. Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. Journal of computational chemistry. 1998 Nov 15;19(14):1639-62.
[8] Nagarajan K, Sharan G, Jeshvanth A, Nightingale T, Dheeneswari P, Sumanthi A. Evaluation of antihistaminic activity of leaves of Acalypha canescana in isolated ileum of rabbit. Int. J. Pharm. Pharm. Sci. 2010;2(2):19-20.
[9] Bennett T. Nerve-mediated excitation and inhibition of the smooth muscle cells of the avian gizzard. The Journal of physiology. 1969 Oct;204(3):669.
[10] Catterall WA. Structure and regulation of voltage-gated Ca2+ channels. Annual review of cell and developmental biology. 2000 Nov;16(1):521-55.
[11] Karaki H, Weiss GB. Calcium release in smooth muscle. Life sciences. 1988 Jan 1;42(2):111-22.
[12] Bolton TB. Mechanisms of action of transmitters and other substances on smooth muscle. Physiological Reviews. 1979 Jul 1;59(3):606-718.
[13] Carl A, Lee HK, Sanders KM. Regulation of ion channels in smooth muscles by calcium. American Journal of Physiology-Cell Physiology. 1996 Jul 1;271(1):C9-34.
[14] Godfraind T, Miller RO, Wibo MA. Calcium antagonism and calcium entry blockade. Pharmacological Reviews. 1986 Dec 1;38(4):321-416.
[15] Ghayur MN, Gilani AH. Pharmacological basis for the medicinal use of ginger in gastrointestinal disorders. Digestive diseases and sciences. 2005 Oct 1;50(10):1889-97.
[16] Fleckenstein A. Specific pharmacology of calcium in myocardium, cardiac pacemakers, and vascular smooth muscle. Annual Review of Pharmacology and Toxicology. 1977 Apr;17(1):149-66.
[17] Eglen RM, Hegde SS, Watson N. Muscarinic receptor subtypes and smooth muscle function. Pharmacological reviews. 1996 Dec 1;48(4):531-65.
[18] Thomas EA, Baker SA, Ehlert FJ. Functional role for the M2 muscarinic receptor in smooth muscle of guinea pig ileum. Molecular pharmacology. 1993 Jul 1;44(1):102-10.

[19] Eglen RM, Reddy H, Watson N, Challiss RJ. Muscarinic acetylcholine receptor subtypes in smooth muscle. Trends in pharmacological sciences. 1994 Apr 30;15(4):114-9.
[20] Bolton TB, Zholos AV. Activation of M 2 muscarinic receptors in guinea-pig ileum opens cationic channels modulated by M 3 muscarinic receptors. Life sciences. 1997 Feb 21;60(13):1121-8.
[21] Eglen RM, Whiting RL. Heterogeneity of vascular muscarinic receptors. Journal of autonomic pharmacology. 1990 Aug 1;10(4):233-46.
[22] Hall JM, Caulfield MP, Watson SP, Guard S. Receptor subtypes or species homologues: relevance to drug discovery. Trends in pharmacological sciences. 1993 Oct 31;14(10):376-83.
[23] Van Zwieten PA, Doods HN. Muscarinic receptors and drugs in cardiovascular medicine. Cardiovascular Drugs and Therapy. 1995 Feb 1;9(1):159-67.
[24] Morini G, Kuemmerle JF, Impicciatore M, Grider JR, Makhlouf GM. Coexistence of histamine H1 and H2 receptors coupled to distinct signal transduction pathways in isolated intestinal muscle cells. Journal of Pharmacology and Experimental Therapeutics. 1993 Feb 1;264(2):598-603.
[25] Kotlikoff MI, Murray RK, Reynolds EE. Histamine-induced calcium release and phorbol antagonism in cultured airway smooth muscle cells. American Journal of Physiology-Cell Physiology. 1987 Oct 1;253(4):C561-6.
[26] Consolini AE, Berardi A, Rosella MA, Volonté M. Antispasmodic effects of Aloysia polystachya and A. gratissima tinctures and extracts are due to non-competitive inhibition of intestinal contractility induced by acethylcholine and calcium. Revista Brasileira de Farmacognosia. 2011 Oct;21(5):889-900.