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Phytochemical analysis and hypotensive potential of *Teucrium stocksianum* Boiss.

Madiha Aziz[1], Alamgeer[1,2]*, Nabeela Noor[1], Muhammad Saleem[2], Hafiz Muhammad Irfan[1], Nasser Hadal Alotaibi[3], Ameeduzzafar[3], Syed Nasir Abbas Bukhari[3] and Pervaiz Akthar Shah[2]

[1]Laboratory of Cardiovascular Research and Integrative Pharmacology, College of Pharmacy, University of Sargodha, Pakistan
[2]Punjab University College of Pharmacy, University of the Punjab, Lahore, Pakistan
[3]College of Pharmacy, Jouf University, Aljouf, Sakaka, Saudi Arabia

Abstract: *Teucrium stocksianum* Boiss. is an aromatic perennial herb. It has long been used traditionally in the treatment of hypertension in northern areas of Pakistan. The aim of this study was to evaluate its folkloric claim as hypotensive plant, phytochemical analysis and to predict potential phytoconstituent through *in-silico* studies. Hypotensive effect was investigated in anesthetized normotensive Sprague–Dawley rats. Recording of chronotropic and inotropic effect of plant extract in isolated right atria was done using tissue organ bath technique. Further, phytochemical characterization was performed through LC-MS. Whereas docking studies were carried out against M2 mAchR and Ca^{2+} Channel receptor. Dose dependent reduction in systolic, diastolic, mean arterial pressure and heart rate was observed. Pretreatment with atropine and amlodipine significantly ($p<0.001$) reduced the hypotensive and negative chronotropic and inotropic effect. Phytochemical studies revealed the presence of twenty active compounds including Luteolin, Sarmentosin epoxide and Quinic acid. Docking studies showed pronounced interactions of majority of these phytochemicals with M2 mAch receptor in agonistic way and Ca^{2+} Channel receptor in antagonistic way. Results speculate that dose dependent hypotensive and bradycardia effect of *Teucrium stocksianum* are mediated through muscarinic pathway and Ca^{2+}antagonism and is also well predicted by *in-silico* studies.

Keywords: *Teucrium stocksianum*, invasive technique, hypotensive, bradycardia, LC-MS.

INTRODUCTION

Hypertension is a major public health problem due to its high prevalence all around the globe. Around 7.5 million deaths or 12.8% of the total of all annual deaths worldwide occur due to high blood pressure. It is predicted that it will be expanded to 1.56 billion adults with hypertension in 2025 (Singh *et al*., 2017). Hypertension is a modifiable and major risk factor for cardiovascular diseases and a "silent killer" due to its high mortality rates and lack of early symptoms (Shah *et al*., 2018). Many synthetic drugs have been widely used for the treatment of hypertension, but herbal medicines still remain a popular choice. The abundant use of these medicinal plants has led to extensive research in this area to determine their potential efficacy and modern cardiovascular drugs are now available as natural products (Sant's Anna *et al*., 2017). Pakistan has plethora of medicinal plants being used by aborigines for the treatment of various ailments including cardiovascular diseases However limited pharmacological investigation are available to document the antihypertensive effect of these plants. *Teucrium stocksianum* Boiss. is member of Lamiaceae family. This aromatic perennial and woody herb is mostly found in the hilly area of northern Oman as well as United Arab Emirates, (Nadaf *et al*., 2003) Pakistan (Ahmad *et al*., 2002) and mountainous area of Iran (Mojab *et al*., 2003). It possess wide spectrum of physiological activities. Literature survey shows that this plant is used in folk medicine for treating diarrhea, cough, jaundice and abdominal pain diabetes and hypertension (Shah *et al*., 2012). Due to its uses in traditional medicine system, several scientific studies have been conducted to rationalize and establish its therapeutic potential. The plant has been reported to have antioxidant, anti-nociceptive, anti-inflammatory, anti-seizure, hepato protective and antimicrobial potential (Shah and shah, 2015). Despite the no of biological activities no study has undertaken the evaluation of antihypertensive effect of this plant. Thus the present study was designed to undertake the investigation of chemical composition, hypotensive effect of aqueous methanolic extract of *Teucrium stocksianum* in normotensive anesthetized rats and also to elucidate the mechanism underlying this effect. Indeed, molecular docking has become one of the valuable tools for studying the interactions of the inhibitors and stimulators at the active site of the receptor (Khan *et al.*, 2019) Therefore an effort has also been made to correlate *in vivo* and *in vitro* studies result with in silico computational studies to make a level of confirmation about mechanism of action of plant extract and link it to its specific phytochemical(s).

**Corresponding author:* e-mail: -alam_yuchi@yahoo.com

MATERIALS AND METHODS

Collection of plant and extraction Process

The plant was collected from Lower Dir, KPK in April-May 2018 in its growing season. The plant sample was identified and authenticated by taxonomist Dr. Aminullah Shah from Department of Botany University of Sargodha-Pakistan. Voucher specimen was deposited in Herbarium of Department of Botany, University of Sargodha for further reference. After shade drying whole plant material was ground to coarse powder and soaked in aqueous methanol solution (70:30) for 72 hours. After 72 hours it was filtered through a porous cloth, the filtrate was collected and plant material was soaked again for 72 hours twice. All of the collected filtrate was combined, again filtered through Whattman qualitative Grade 1 filter paper and then concentrated in a rotary evaporator to obtain a thick crude extract (Ghayur *et al*., 2005).

Measurement of blood pressure in normotensive rats

Sprague-Dawley rats (220-250g) of a local strain housed in precisely maintained environment (12h light/dark cycle, 25±1°C temperature) at animal house of University of Sargodha fed with standardized pellet feed and water ad libitum were used in various experimental protocols. The study design was approved by Animal Ethical Committee of University of Sargodha (No.IAEC/UOS/ 2018/57).

A pressure transducer coupled to Power Lab recording system with an application program (Chart, v 6.1; all from ADI Instruments; Castle Hill, Australia) was used for blood pressure (BP) and heart rate (HR) measurement. The animals were anaesthetized with an intra peritoneal injection of sodium thiopental (70-90mg/kg body weight).The invasive BP measurement protocol as explained Younis *et al*., 2020 was followed with slight modifications. The animals were allowed to stabilize for 30 min before analysis and then dose-response relationship to *Teucrium stocksianum* aqueous methanolic extract (10-160 mg/kg) was determined intravenously followed by flushing in with 0.1 ml saline.

Evaluation of possible hypotensive mechanisms of action of Teucrium stocksianum

Animals were anesthetized and subjected to blood pressure measurement following the aforementioned procedure for elucidation of underlying mechanisms of hypotensive effect of plant extract for this purpose various agonists and antagonists such as atropine (1mg/kg), propranolol (1mg/kg), NG-nitro-L-arginine methyl ester (L-NAME, 20mg/kg) sodium nitropuroside 2.5mg/kg, captopril 2.5mg/kg, amlodipine (1mg/kg) and hexamethonium bromide 30mg/kg were used (Shih *et al*., 2008).Each standard drug was given 10min prior to plant extract 80mg/kg administration which is representative dose that produced 40-50% of reduction in blood pressure. Change in SBP and HR shown by intravenous injection of plant extract was evaluated for 40 minutes after treatment.

In vitro study of Inotropic and chronotropic effect of Tsk

Experimental protocol

The inotropic and chronotropic effects of Tsk on myocardial contractile performance were evaluated on rat isolated right atrial muscle strips of normotensive SD rats, by following the method of Aslam *et al* (2016) with slight modifications. The force and rate of spontaneous contractions of atria was recorded under 1.0 g tension by isometric transducer connected to Power Lab data acquisition system (AD Instruments, Australia). An equilibration period of 30 min was allowed with fluid changes at 10 min intervals before addition of test substance. Percentage changes in rate and force of baseline contractions were calculated after addition of test substance in cumulative doses.

Phytochemical analysis

To probe the bioactive constituents of the crude extract through RP UHPLC-MS, the method as explained by Saleem *et al*., 2019 was adopted with Chromatographic System specifications were as; Column: Agilent Zorbax Eclipse XDB-C18, narrow-bore 2.1×150mm, 3.5µm (P/N: 930 990 -902) Injection Volume: 20µL Flow rate: 0.5ml/min Concentration (Sample/Standard): 1.0µL. 0.1% formic acid in water (A) Diluents: Mobile Phase; 0.1% formic acid in acetonitrile (B).Whereas, obtained results were processed with Agilent Mass Hunter Qualitative Analysis B.05.00 (Metabolomics-2017- 00004.m). Whereas to identify the compounds Search Database: METLIN_AM_PCDL-Ne 170502.cdb, with parameters as: Match tolerance: 5ppm, Positive Ions: +H, +Na, +NH4, Negative Ions: H. was used.

In-silico study

The 20 chemically diverse phytochemical constituents obtained from phytochemistry of plant extract were considered as test set (TS) for molecular docking against various CVS targets notably as predicted by *in-vivo* and *in-vitro* experimentation outcomes. To make correlation between *in-vivo, in-vitro* and *in-silico* studies, two CVS targets such as M-2 mAch receptor and Ca^{2+} Channel receptor were selected for carrying out molecular docking studies.

Molecular docking

The docking study protocol as explained by Gul *et al*., 2019 was followed using MOE-Dock. The Crystal Structure of Ca^{2+} Channel receptor protein and M2 mAchR protein was taken from server using PDB ID entry 1T0J (M Iman *et al.*, 2011) and 4MQS (Fish I *et al*., 2017) respectively. For detailed insight into different binding interactions between the best docked

Table 1: Effect of graded doses of *Teucrium stocksianum* on blood pressure and heart rate

Parameter	Control	10mg/kg	20mg/kg	40mg/kg	80mg/kg	160mg/kg
SBP	115.20±2.7	89.99±4.5 (21.88)	69.78±10.8** (39.42)	66.50±12.5** 42.27%	63.38±11.8*** 44.98%	52.66±6.9*** 54.28%
DBP	101.52±3.9	79.63±5.9 (21.56)	60.07±11.8** (40.82)	50.06±5.2*** (50.68)	48.01±5.8*** (52.70)	36.51±4.2*** (64.04)
MAP	111.87±6.6	77.83±1.4* (30.42)	61.67±8.2*** (44.87)	56.99±9.0*** (49.05)	55.34±8.2*** (50.70)	45.44±5.8*** (59.38)
HR	369.27±12.3	354.63±12.5 (3.96)	315.28±6.2 (14.62)	311.77±5.8 (15.57)	286.53±8.1 (22.40)***	269.03±7.9 (27.14)***

Results are expressed as mean ± SEM. Values in parenthesis denotes percentage reduction in each parameter *p<0.05, **p<0.01, ***p<0.001 compared with control. n = 5.

Table 2: List of compounds identified by LC-MS analysis of TSK

S. no	RT(min)	Base Peak m/z	Peak height	Name	Molecular formula	Molecular Weight
1.	0.627	294.0886	60754	Mebendazole	C16 H13 N3 O3	295.0952
2.	0.74	133.0149	311645	3,3-Dimethyl-1,2-dithiolane	C5 H10 S2	134.0224
3.	0.84	290.0886	61630	Sarmentosin epoxide	C11 H17 N O8	291.0959
4.	0.91	128.0354	55132	N-Acryloylglycine	C5 H7 N O3	129.0428
5.	1.737	191.0567	149957	Quinic acid	C7 H12 O6	192.064
6.	6.796	525.1381	52807	Phloroacetophenone 6'-[xylosyl-(1->6)-glucoside]	C21 H30 O13	490.1686
7.	7.687	405.1422	49808	SerTrp Asp	C18 H22 N4 O7	406.1497
8.	8.705	769.2544	1891671	Leonoside A	C35 H46 O19	770.2618
9.	9.44	423.1716	234874	Met PheGln	C19 H28 N4 O5 S	424.1791
10.	10.413	425.1805	49747	gamma-CEHC Glc	C21 H30 O9	426.1876
11.	10.44	407.1703	67670	Vemoflexuoside	C21 H28 O8	408.1779
12.	10.577	285.0416	56335	5,7,2',3'-Tetrahydroxyflavone	C15 H10 O6	286.049
13.	10.599	377.1622	63302	Gibberellin A66	C20 H26 O7	378.1695
14.	10.599	397.1419	63930	N-Feruloylglycyl-L-phenylalanine	C21 H22 N2 O6	378.1695
15.	10.837	407.1733	158185	TrpGlyPhe	C22 H24 N4 O4	408.1806
16.	11.064	407.1716	393078	3-O-Methylniveusin A	C21 H28 O8	408.1787
17.	11.064	407.1716	502760	3-O-Methylniveusin A	C21 H28 O8	408.1787
18.	11.276	509.2058	65323	GW 409544	C31 H30 N2 O5	510.2131
19.	11.762	287.2233	90000	9,16-dihydroxy-palmitic acid	C16 H32 O4	288.2306
20.	11.902	449.185	114537	Copalyldiphosphate	C20 H36 O7 P2	450.1922
21.	12.324	221.1192	123631	(6S)-dehydrovomifoliol	C13 H18 O3	222.1265
22.	12.355	313.0732	203710	Luteolin 5,3'-dimethyl ether	C17 H14 O6	314.0804
23.	13.141	293.1786	51924	Tetradecyl sulfate/sodium tetradecyl sulphate	C14 H30 O4 S	294.1861
24.	15.023	293.2136	158078	α-9(10)-EpODE	C18 H30 O3	294.2208
25.	15.704	295.2291	196306	12-oxo-10Z-octadecenoic acid	C18 H32 O3	296.2362

conformation of test compounds in the active site of protein 3D and 2D ligand plots, ligand-protein interaction diagrams were generated of each compound which showed the key amino acids involved in the binding.

STATISTICAL ANALYSIS

Data obtained from all experiments in current study was expressed as mean ± S.E.M. Results were statistically analyzed using one-way ANOVA followed by Dunnett's post test. P values less than 0.05 were considered significant. GraphPad prism 8.0 was used for statistical analysis.

RESULTS

Hypotensive effect in anesthetized normotensive rats

In anesthetized rats the recorded baseline blood pressure was 115.20±2.7mmHg after 20 minute period of stabilization .In comparison to saline control intravenous administration of aqueous methanolic extract of *Teucrium stocksianum* resulted in immediate fall in MAP that was observed in dose dependent manner. Heart rate was decreased at higher dose. At doses 10, 20, 40, 80,160 mg/kg Tsk reduced the MAP by 21.88±07, 39.42±21, 42.27±11, 44.98±13 and 54.2 8±26% respectively (table 1).

Mechanism underlying the hypotensive activity of Teucrium stocksianum

As shown in fig. 1, the pretreatment of anaesthetized Sprague Dawley normotensive rats with atropine sulphate (1mg/kg) and amlodipine (1mg/kg) significantly ($p<0.05$) reduced the hypotensive effect of the plant extract. While L-NAME (20mg/kg), propanolol 1mg/kg, hexamethonium 30mg/kg, sodium nitropuroside 2.5mg/kg, captopril 2.5mg/kg did not altered the magnitude of hypotensive response of plant extract.

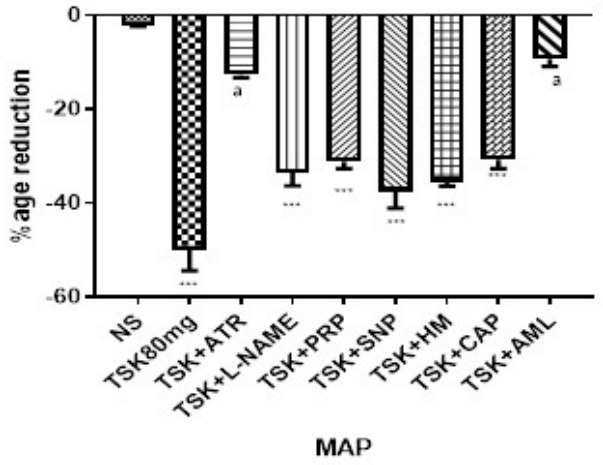

Fig. 1: Effect of aqueous methanolic extract of Teucrium stocksianum (Tsk, 80mg/kg) on Mean arterial blood pressure (MAP) in the presence of various antagonists. Results are indicated as mean ± S.E.M. where *** =$p<0.001$ when compared to control (Normal saline treated group) while a = $p<0.001$ when compared to treated control (Tsk, 80mg/kg). All data are subjected to one-way ANOVA followed by Dunnett's posttest).Where ATR= atropine, PRP= propranolol, SNP sodium nitropruside, HM= hexamethonium, CAP=captopril, AML=amlodipine.

In vitro Inotropic and chronotropic effect of Tsk

Fig. 2A depicts the chronotropic and inotropic effects of Tsk treatment on rat isolated spontaneously beating right, atrial muscle strip, respectively. Tsk reduced the spontaneous beating of the right atrial muscle preparations (negative chronotropy) and force of myocardial contraction negative inotropy) in a concentration-dependent manner To assess whether the negative chronotropic and inotropic effects were mediated through activation of cholinergic receptors, the right atrial muscle preparation was pre-treated with atropine sulphate (ATR, 1µM), a cholinergic receptor antagonist. Pre-treatment of the atrial preparations with ATR resulted in complete abolition of the negative chronotropic and inotropic effect of Tsk (fig. 2B, 2C)

Phytochemical analysis of Teucrium stocksianum

Several compounds were revealed by LC-MS analysis of aquous methanolic extract of *Teucrium stocksianum* twenty compounds were identified and they belong to flavonoid, phenol, terpenoids and fatty acids. As shown in table 2 and fig. 3 Sarmentosin epoxide, Phloroacetophenone 6'-[xylosyl-(1->6)-glucoside], Leonoside A, Copalyldiphosphate,9,16-dihydroxy-palmitic acid and Luteolin 5,3'-dimethyl ether were dominant compounds identified in Tsk.

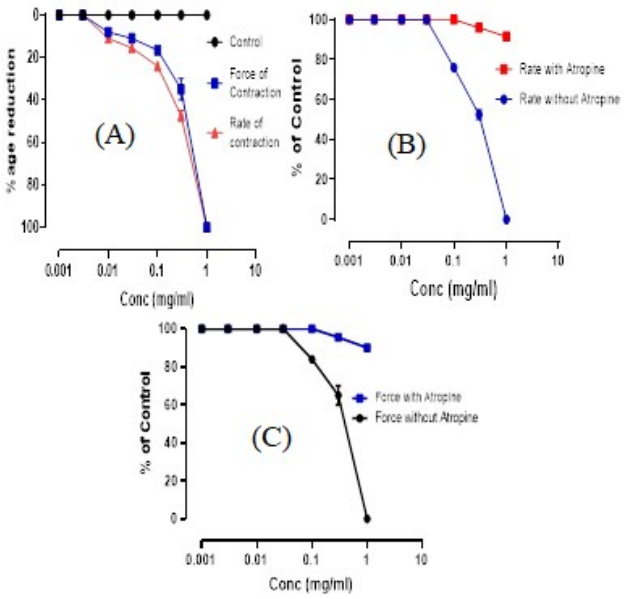

Fig. 2: Concentration response curves (A) show the effect of plant extract on spontaneous rhythmic rate of contraction and force of contraction in isolated SD rat right atrial preparation. While (B) and (C) depicts the effect in the presence and absence of atropine. Values are shown as Mean ± SEM (n=4).

Molecular docking

All the test compounds were docked into the active site of 1T0J (fig. 4) and 4MQS (fig. 5) using the MOE docking method to determine their most suitable docking conformations. Out of 20 test compounds Quinic acid, 5,7,2',3'-Tetrahydroxyflavone Sarmentosin epoxide, Tetradecyl sulfate/sodium tetradecylsulphate, 12-oxo-10Z-octadecenoic acid, Gibberellin A66, gamma-CEHC Glc, Phloroacetophenone 6'-[xylosyl-(1->6)-glucoside] and Copalyldiphosphate showed good antagonistic ligand-protein interaction with Ca^{2+} channel protein. Fig. 6 (A) and fig. 6 (B) represents 3D and 2D diagrams of ligand-L Type Ca^{2+} Channel protein interaction diagram of best docked conformation of test compound Phloroacetophenone 6'-[xylosyl-(1->6)-glucoside] depicting the key amino acids involved in the binding. Whereas, test compounds 5,7,2',3'-Tetrahydroxyflavone, Sarmentosin epoxide, Tetradecyl sulfate/sodium tetradecylsulphate, α-9(10)-EpODE, Gibberellin A66, gamma-CEHC Glc, Leonoside A and Copalyldiphosphate hold considerable agonistic interaction with the active site of M2 receptor protein. Fig. 7 (A) and fig. 7 (B) represents 3D and 2D diagrams of ligand-M2 receptor

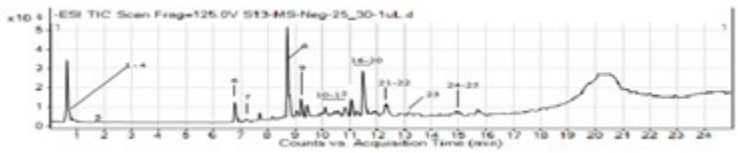

Fig. 3: Chromatogram obtained from LC-MS analysis

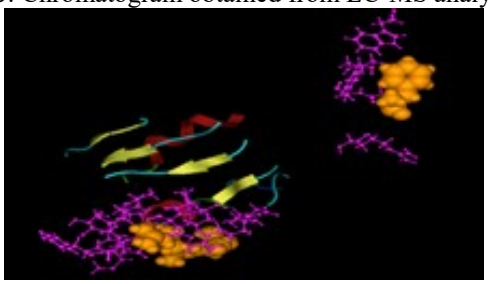

Fig. 4: Active site of Ca2+ channel protein(PDB ID 1T0J)

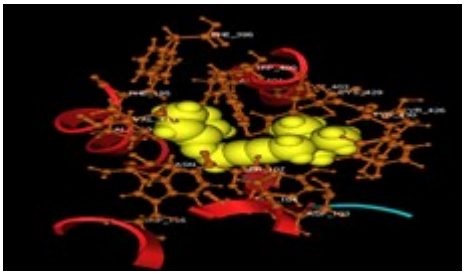

Fig. 5: Active site of M2 receptor (PDB ID 4MQS)

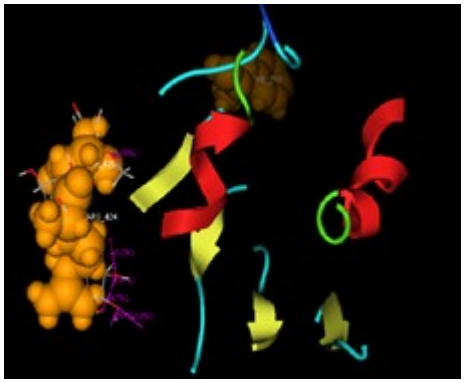

Fig. 6(A): 3D interaction of best docked pose of phloroacetophenone 6'-[xylosyl-(1->6)-glucoside] with L-type calcium channel.

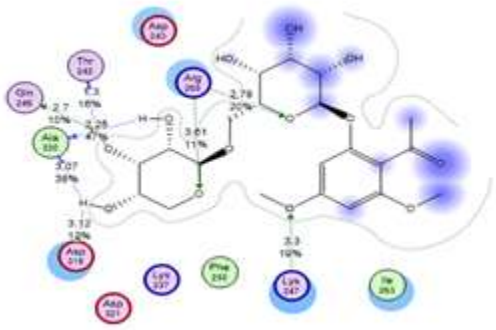

Fig. 6(B): 2D interaction of best docked pose of phloroacetophenone 6'-[xylosyl-(1->6)-glucoside] with L-type calcium channel.

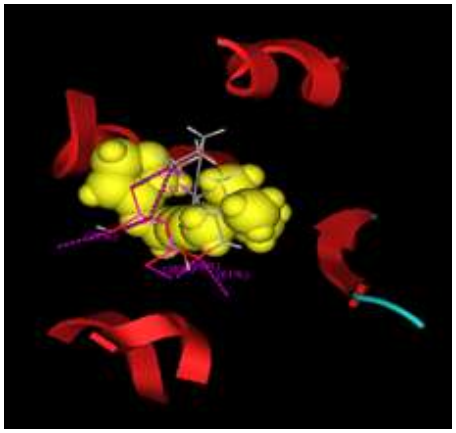

Fig. 7(A): 3D interaction of best docked poses of gamma-CEHC Glc with M2–Receptor(PDB-ID 4MQS).

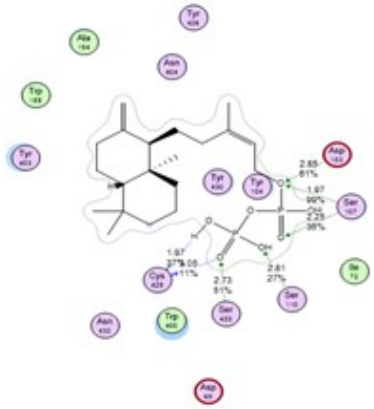

Fig. 7(B): 2D interaction of best docked poses of gamma-CEHC Glc with M2–Receptor (PDB-ID 4MQS).

protein interaction diagrams of best docked conformation of test compound gamma-CEHC Glc highlighting the key amino acids involved in the binding.

DISCUSSION

The research involving the natural products to obtain new antihypertensive drugs has shown a significant growth in the last decade. Even in the presence of other drug discovery methods the value of traditional medicine cannot be snubbed. One of the promising area of research in which plant source has contributed massively is cardiovascular research (Alamgeer *et al*., 2014). *Teucrium stocksianum* has been traditionally used for the treatment [of hypertension (Ahmad *et al*., 2015). The aim of this study was to look in the potential activity of *T.*

stocksianum in hypertension treatment. For this purpose Invasive blood pressure monitoring technique that allowed observing the effect of extract directly by injecting into the systemic circulation was used. Intravenous injection of the aqueous methanolic extract of *T. stocksianum* caused a dose-dependent fall in SBP, DBP, MAP and HR in normotensive rats. Further investigation about the possible underlying mechanism responsible for this hypotensive effect various agonist and antagonists were administered before the extract. The results of in-*vivo* study revealed that L-NAME, hexamethonium, sodium nitropruside, propranolol and captopril have not altered the hypotensive response of plant extract thus excluding the role of nitric oxide pathway, adrenergic, ganglion blockage and RAAS pathway. In the presence of Atropine, an antagonist of muscarinic receptors, hypotensive action of *T. stocksianum* was significantly reduced when compared to the control which suggests the existence of cholinomimetic components in the extract. These substances may act according to the same mechanism as acetylcholine (ACh). Blockage of calcium influx through L-type calcium channels may also be involved in the *T. stocksianum* induced hypotension as suggested by its sensitivity to amlodipine an L-type Ca^{2+} channel blocker. We further aimed to investigate the *in-vitro* chronotropic and inotropic effect of extract. In isolated right atrial preparation both force and rate of contraction were decreased by extract at higher doses. Pretreatment of atria with atropine completely abolished the negative chronotropic and inotropic effect of extract. Thus, we speculate that cardio-inhibitory effect of extract is due to activation of M2 receptors and inhibition of calcium channels. LC-MS analysis of aqueous methanolic extract of *Teucrium stocksianum* identified the 20 compounds.The LC-MS identified 20 phytochemicals of plant extracts were subjected to molecular docking studies. Two receptor proteins including M2 mAch *Receptor and L-Type* Ca^{2+} *Channel Receptor were* selected keeping in mind *in-vivo* and *in- vitro* results of plants extract to get a predictive idea of the possible phytochemicals involve in manifestation of hypotensive response. Out of 20 test compounds Quinic acid, 5,7,2',3'-Tetrahydroxyflavone, Sarmentosin epoxide, Tetradecyl sulfate/sodium tetradecylsulphate, 12-oxo-10Z-octadecenoic acid Gibberellin A66, gamma-CEHC Glc, Phloroacetophenone 6'-[xylosyl-(1->6)-glucoside] and Copalyldiphosphate showed good antagonistic ligand-protein interaction with L-Type Ca^{2+} channel protein. The best docked poses of the test compounds showed to superimpose innate ligand with amino acids as Phe 250, Asp 243, Lys 237, Arg 265, Asp 319, Ala 320, Gln 246, Gly 238, Thr 242 playing major role in Ligand-protein interaction depicted in fig. 6B.These results corresponds well with the studies done by (Maryum *et al*., 2011; Estella G. da Mota *et al.,* 2014; Choudhari *et al.,* 2013). Keeping in view the above detailing, test compounds 5,7,2',3'-Tetrahydroxyflavone, Sarmentosin epoxide, Tetradecyl sulfate/sodium tetradecylsulphate, α-9(10)-EpODE, Gibberellin A66, gamma-CEHC Glc, Leonoside A and Copalyldiphosphate best docked poses showed considerable wander vall interactions with active site of M_2 mAchR proteins. Similarly test compounds seems to superimpose bound ligand with residues such as Asp 103, Ser 107, IIe 72, Ser 110, Tyr 104, Tyr 430, Cys 429, Ser 233, Trp 400, Asp 69 and Asn 432 playing major role in Ligand –protein agonistic interaction (fig. 7B) Almost all test compounds interact well with aspartate residue Asp 103 making ligand-receptor stabilized complex which is in consistent with the study carried out by Vistoli *C,* 2008 and Fish I *et al*., 2017. These findings suggest that *in-vivo* study results predicted Ca^{2+} Channel antagonistic activity and muscarinic agonistic activity of plant extract can be due to above detailed test compounds (phytochemicals) showing their promising *in-silico* activity against docked receptor proteins.

CONCLUSION

This study provides the scientific validation for traditional use of *Teucrium stocksianum* in the treatment of hypertension. Moreover isolated phytochemicals showing Ca^{2+} channel antagonistic and M_2 receptor agonistic property in docking study should be subjected for *in vivo* study to further confirm the chemical constituents responsible for hypotensive potential of plant. This would result in potential candidate for drug development of new herbal medicine for the treatment of hypertension.

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