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Ali Rizwan
Faculty of Pharmacy, The Islamia University of Bahawalpur, Pakistan

Rao Saeed
Faculty of Pharmacy, The Islamia University of Bahawalpur, Pakistan

Taseer Ahmadv
Shifa College of Pharmaceutical Sciences, Shifa Tameer-e-Millat University, Islamabad, Pakistan

Aftab Ullah
Faculty of Pharmacy, The Islamia University of Bahawalpur, Pakistan

Zaira Ather
Faculty of Pharmacy, University of Sargodha, Sargodha, Pakistan

Sohail Muhammad
Shifa College of Pharmaceutical Sciences, Shifa Tameer-e-Millat University, Islamabad, Pakistan

Iradat Hussain
Shifa College of Pharmaceutical Sciences, Shifa Tameer-e-Millat University, Islamabad, Pakistan

Usman Ali
Faculty of Pharmacy, The Islamia University of Bahawalpur, Pakistan

Correspondence:
Taseer Ahmad
Shifa College of Pharmaceutical Sciences, Shifa Tameer-e-Millat University, Islamabad, Pakistan
Email: drtasir2011@gmail.com

Evaluation of *Salvia haematodes* Linn for their anti-oxidant, anti-microbial, anti-lipoxygenase and anti-cholinesterase activity

Ali Rizwan, Rao Saeed, Taseer Ahmad, Aftab Ullah, Zaira Ather, Sohail Muhammad, Iradat Hussain, Usman Ali*

Abstract

The indigenous medicinal plant *Salvia haematodes* Linn (SAL) was screened for its pharmacological activities. The crude extract fractions were prepared in methanol, n-hexane, di chloromethane (DCM) and n-butanol by cold maceration process. The antioxidant effect of the extract fractions was observed by 1-1- diphenyl 2-picryl hydrazine (DPPH) radical scavenging activity. The anti-microbial activity was observed by using disc diffusion method. Furthermore, anti-enzymatic activity was studied against LOX (anti-lipoxygenase), acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The results exhibited that the n-hexane (0.5 mg/mL) and DCM (0.5 mg/mL) fractions of SAL showed 83.3 ± 0.8% and 91.56 ± 0.7 % inhibition of DPPH radical. Whereas, the n-butanol fraction of the crude extract did not show any effect. The methanolic extract (10 mg/disc) of SAL inhibited the growth of *E. coli* at 12±0.5 mm as compared to ciprofloxacin (17.3 ± 0.5 mm). Moreover, at concentration of 0.5 mg/mL, the n-hexane and DMC extracts of SAL inhibited 68.19±0.39 % and 75.8 ± 1.7% of LOX enzyme respectively, against standard Baicallin (93.7 ± 1.2 %). The methanol fraction of SAL showed 77.95 ± 0.6 % of AChE inhibition, whereas, Eserine standard inhibited 91.2 ±1.1% of AChE. The methanolic fraction of SAL showed 51.9±0.4 % inhibition of BChE enzyme against standard Eserine (82.8±1.0%). However, the DCM and n-hexane fractions did not elucidate any activity. Therefore, it is concluded that SAL have different pharmacology activities that may be due to presence of certain pharmacologically active compound.

Keywords: *Salvia haematodes* Linn, Anti-oxidant, Antimicrobial, LOX, AChE and BChE.

Introduction

The drugs from plants are subjected to the human and animal testing for determining their safety and to evaluate their potency.[1] Ethno pharmacological exploration of the natural medicinal plants used by different people around the world for various purposes may help in finding newer and cost effective drugs, which can be further used for various purposes. So the screening of medicinal plants may help to find us compounds of potential medicinal activities that can be used for various purposes.[2]

In-vitro studies of the plant extracts or drugs are more economical and cheap. It also involves little or no animal testing, besides this; it can provide an output of testing in a very suitable time. In this way, a great number of extracts can be evaluated for the discovery of newer molecules and make advances in the scientific research.[3]

Recent developments in the field of biomedicine have shown that many diseases are caused by the formation of free radicals and reactive oxygen species. Brain dysfunction, cancer, arthritis, heart problems and immune related diseases are the examples of the major diseases which are caused due to the free radical formation in the living body.[4] Natural herbs and plants have played a vital role in preventing the oxidation of the cellular components like *Asparagus* laricinusandAmberboadivaritica.[5,6] The agents used to treat bacterial diseases are referred to as antibiotics which are very effective and potent chemicals obtained from micro-organisms and are also prepared synthetically. These must be used carefully as bacteria may develop resistance against these drugs and make them ineffective. Naturally occurring medicinal plants are used in many regions of the world for their antibacterial activities. Some of the reported plants like *Sarcochlamy pulcherrima* and *Asparagus laricinus* have antibacterial activity.[5, 7]

Furthermore, enzymes are the very active protein molecules that have the capability to catalyze chemical and biochemical reactions and act like biological catalysts. Enzyme inhibitors are mostly used as drugs in various pathological conditions. For example, the NSAIDs like aspirin can stop the cyclo-oxygenase 1 (COX-1) and cyclo-oxygenase 2 (COX-2) enzymes that are involved in inflammation. Certain medicinal plants have the property to inhibit various enzymes so they may be very useful in preventing the enzyme related diseases and problems. For instance, certain plants have the ability to inhibit the cholinesterase enzymes like AChE and BChE.[8-10] Moreover, inflammation is considered to be primary defense physiologic mechanism which helps the body for protecting it against burn, infection, toxic chemicals, allergens and other harmful stimuli. If inflammation is left uncontrolled and persistent, it will act as an etiologic factor for many other chronic diseases.[11] Plants reported for their anti-inflammatory activity are *Xeromphis spinosa* and *Foeniculum vulgare.*[12, 13]
Salvia haematodes Linn is an herbaceous perennial in the family Lamiaceae. The extracts of Salvia species exhibit anti-inflammatory, anti-malarial, anti-microbial and anti-oxidant activities. These studies provide a scientific foundation to authorize the use of these Salvia species in the conventional medicine.[14]
Therefore, it is of great interest to carry out a screening of plants, in order to validate their use in folk medicine and to reveal the active principle by isolation and characterization of their constituents.

Materials and Methods

Plant Selection

For the pharmacological screening, SAL was bought from the herbal market of Bahawalpur City. The plant was given to the taxonomist, Prof. Tayyeb Qureshi of The Punjab College Bahawalpur for identification. The plant after identification was submitted to the Herbarium of The Islamia University of Bahawalpur. The voucher number of SAL was SH-RN-06-048.

Extract Preparation

The grinding of plant material was carried out into small coarse particles, not larger than black pepper particles. 500 g of the ground material was taken and soaked in 80% methanol for one week with regular shaking and stirring at room temperature. Filtration was carried out after one week through muslin cloth and then with Whattman qualitative grade 1 filter paper. The above procedure of filtration was repeated for a total of three times. The filtrate was collected from all the filtrations and the solvent was evaporated under reduced pressure, using rotary evaporator (Laborota 4000-effieicient Heidolph, Germany) at 40-60 °C. A semisolid mass was obtained which was stored in oven at 20-25 °C. After that, different fractions of the plant were prepared from the obtained semisolid mass. Four solvents of different polarities were used (like n-hexane, dichloromethane and n-butanol and methanol). The crude plant extract was allowed to dissolve in these four solvents by sonication.

Phytochemical investigation

The crude extract was tested for phytochemical constituents like alkaloids, phenols, cardiac glycosides, saponinsetc by using different standard methods.[15]

Pharmacological Investigation

Anti-oxidant activity of SAL

10 µL of crude extract and 90 µL of methanolic DPPH (100 µM) were added in a 96-well plate. The contents were allowed to mix and stand for about 30 minutes at room temperature. The absorbance was taken at 517 nm by taking Quercetin as a standard. DPPH radical scavenging activity is the most extensively used antioxidant test used for determining the antioxidant potential of plants. It can react with the compounds which can donate a Hydrogen atom. The compounds which have the capability to give a Hydrogen atom to DPPH are called antioxidants or radical scavengers. In an assay, the discoloration of the violet color shows the radical scavenging property of the antioxidant substance.[16]

Anti-microbial activity of SAL

For testing the anti-microbial activity of crude plant extracts, a Gram negative bacterium *E. coli* was selected. The bacterial strains were obtained from the Department of Biochemistry, The Islamia University of Bahawalpur. Strains of bacteria were vulnerable to most of the antibiotics that are used in clinical practice like Ciprofloxacin, Gentamicin and Ampicillin. Nutrient broth was used for the proliferation of bacterial cultures. The composition of the broth is given in the Annexure. Bacteria were allowed to grow in nutrient broth for 18 hours at 37 °C. Dilution of bacterial culture was made by sterile nutrient broth up to $1x10^8$ colony forming units (CFU) and its density was compared with 0.5 McFarland standard. 10 mL of solvent was taken and 10 mg of plant extract was mixed with it. 6 mm sized sterile filter paper discs were cut and each disc was impregnated with 1 mL of above extract solution in 10 µl portions. In this way each disc had 10 µg of plant extract. The discs were allowed to dry at room temperature. A standard antibiotic Ciprofloxacin (5µg) was purchased from Oxford Ltd., England. Nutrient agar was used for the growth of bacterial cultures. The composition of broth is given in the Annexure. About 20 g of nutrient agar was dispersed in 1 liter of water and then it was allowed to autoclave at 21 °C for 15 minutes at a pressure of 15 psi. After autoclaving, the media was allowed to cool up to 60°C. After that 20-25 mL was poured in petri dishes and allowed to solidify at room temperature. The plates were stored at 4°C after solidification.[17- 19]

Anti-inflammatory activity of SAL

Anti-inflammatory activity was assayed according to the method described by Baylac *et al.*, 2003 with slight modifications.[20] A volume of 200 µL lipoxygenase test mixture was taken, containing 140 µL sodium phosphate buffer (100 mM, pH 8.0), 20 µL of test compound and 15 µL of purified lipoxygenase enzyme (600 units well-1, Sigma Inc.). The contents were allowed to mix and absorbance was taken at 234 nm and then incubated for 10 minutes at 25°C. The reaction was initiated by adding 25 µL of substrate solution. The change in absorbance was observed after 6 min at 234 nm using 96-well plate reader Synergy HT, Biotek, USA. The positive and negative controls were included in the assay. Baicalin (0.5 mM well-1) was used as a positive control. An

IC_{50} value was calculated using EZ–Fit Enzyme Kinetics software (Perrella Scientific Inc. Amherst, USA). [20]

AChE inhibitory activity of SAL

The AChE inhibition activity was performed according to the method as described by Birman, 1985 with slight modifications.[21] Total volume of the reaction mixture was 100 µL, containing 60 µL Na_2HPO_4 buffer with concentration of 50 mM and pH 7.7,to this mixture10 µL test compound (0.5 mM well-1) was added, followed by the addition of 10 µL (0.005 unit well-1) enzyme. The contents were mixed thoroughly and absorbance was taken at 405 nm. The contents were allowedto incubate for 10 min at 37ºC. The reaction was initiated by the addition of 10 µL of 0.5 mM well-1 substrate (acetylthiocholine iodide), followed by the addition of 10 µL DTNB (0.5 mM well-1). After 30 min of incubation at 37ºC, absorbance was taken at 405 nm using 96-well plate reader Synergy HT, Biotek, USA. All experiments were carried out with their respective controls. Eserine (0.5 mMwell-1) was used as a positive control. IC_{50} values were calculated using EZ–Fit Enzyme kinetics software (Perrella Scientific Inc. Amherst, USA).[21,]

BChE assay of SAL

The BChE inhibition activity was performed according to the method as described by Birman, 1985 with slight modifications.[21] Total volume of the reaction mixture was 100 µL which contained 60 µL, Na_2HPO_4 buffer, to this mixture 50 mM and pH 7.7,10 µL test compound 0.5 mM well-1 was added followed by the addition of 10 µL (0.5 unit well-1) BChE (Sigma Inc.). The contents were allowed to mix and absorbance was taken at 405 nm and then allowed to incubate for 10 min at 37ºC. The reaction was initiated by the addition of 10 µL of 0.5 mM well-1 substrate (butyrylthiocholine chloride). Followed by the addition of 10 µL DTNB, 0.5 mM well-1. After 30 min of incubation at 37ºC, absorbance was measured at 405 nm using 96-well plate reader Synergy HT, Biotek, USA. All experiments were carried out with their respective controls. Eserine (0.5 mM well-1) was used as positive control. IC_{50} values were calculated using EZ–Fit Enzyme kinetics software (Perrella Scientific Inc. Amherst, USA).[21]

Results

Phytochemical investigation

The phytochemical analysis of SAL was carried out according to the given protocols and the following chemicals were found to be present in the SAL (Table 1).

Table 1: Phytochemical analysis of SAL

Chemical Constituent	Present/Absent
Alkaloids	Absent
Tannins	Absent
Saponins	Absent
Anthraquinones	Absent
Glycosides	Absent
Flavonoids	Present
Terpenoids	Present
Steroids	Present
Carbohydrates	Present
Ketones	Absent
Pentoses	Present
Soluble starches	Present
Phenols	Present

Anti-oxidant activity of SAL

At a concentration of 0.5 mg/mL, SAL crude extract in dichloromethane solvent scavenged 91.56±0.7 % of DPPH radical and n-hexane fraction of the extract showed 83.33± 0.87 % scavenging of DPPH radical. The n-Butanol fraction of the plant did not show any prominent activity against DPPH, while, at the same concentration Quercetin caused 93.21±0.97 % inhibition (Table 2).

Table 2: Antioxidant activity of SAL

Plant	n-hexane fraction	DCM fraction	n-butanol fraction
SAL	83.3±0.8	91.5±0.7	-
Quercetin	93.2±0.9%	-	-

Anti-microbial activity of SAL

The aqueous methanolic extract of SAL was tested against Gram negative bacteria, Escherichia coli at a concentration of 10 mg/disc as shown in Table 3. The SAL inhibited the growth of E. coli, the results of anti-microbial action of crude extract of SAL and a standard antibiotic Ciprofloxacin are shown in table 3.

Table 3: Antimicrobial activity of SAL

Sr. No	Organism	Zone of Inhibition Diameter (mm)	
		SAL Extract (Methanol)	Ciprofloxacin
1	*E. coli*	12±0.5	17.3±0.5
		SAL Extract (Methanol)	Ciprofloxacin
2	*E. coli*	10.2±0.1	17.1±0.5

Results are represented as mean ± standard deviation.

Anti-Inflammatory activity of SAL

At a concentration of 0.5 mg, the dichloromethane fraction of SAL showed 75.83±1.74 % inhibition of LOX whereas the n-hexane fraction of crude extract of SAL showed 68.19±0.39 % inhibition of LOX enzyme at 0.5 mg concentration of LOX enzyme against Baicalin standard which showed 93.79±1.27 % inhibition of LOX at the same concentration. The IC50 value was found to be <400 µg/L and that of standard Baicalin was 22.14± 0.08 (Table 4).

Table 4: Anti-Inflammatory activity of SAL

µg/L	Conc./well	n-hexane fraction	DCM fraction	n-butanol fraction	IC_{50} µg/L
SAL	0.5 mg	68.1±0.3 %	75.8±1.7 %	-	<400
Baicalin	0.25 mM	93.7±1.2 %	-	-	22.1 ± 0.08

Results are represented as mean ± standard deviation. DCM= Dichloromethane

AChE inhibitory activity and BChE assay of SAL

The methanolic crude extract of SALat a concentration of 0.5 mg inhibited 77.95±0.69 % of AChE,whereas 51.95±0.45 % of BChE enzyme inhibition was observed. On the other hand, the Eserine control inhibited 91.29±1.17 % of AChE at 0.25mM amount and 82.82±1.09 % inhibition of BChE was seen with Eserine control at the same concentration (Table 6). The IC_{50} value for AChE was found to be <400 µg/L while that of Eserine standard was 0.04±0.001 µM/L. Whereas, IC_{50} in case of BChE was <400 µg/L while that of Eserine standard was 0.85±0.09 µM/L (Table 5).

Table 5: AChE activity of SAL

AChE Inhibition					
Plant Extract	Conc./well	methanol fraction	DCM-fraction	n-hexane fraction	IC_{50} (µg/L)
SAL	0.5 mg	77.9±0.6 %	-	-	<400
Eserine	0.5 mM	91.2±1.17 %	-	-	0.04± 0.001
BChE Inhibition					
SAL	0.5 mg	51.9±0.45 %	-	-	<400
Eserine	0.25 mM	82.8±1.09 %	-	-	0.85± 0.09

Results are represented as mean ± standard deviation. DCM= Dichloromethane

Discussion

Plant materials remain an important resource to combat serious diseases in the world. The traditional medicinal methods, especially the use of medicinal plants, still play a vital role to cover the basic health needs in the developing countries.[22]

SAL exhibited a significant antioxidant activity by inhibiting DPPH radical. The antioxidant effects of the plants may be due to the presence of the phenolic compounds that have the ability to interact with the agents that can gain hydrogen atom and get reduced, thus preventing oxidation to take place. Most phenolic compounds have antioxidant actions when used in the humans for medicinal purposes.[23]

Similarly, SAL revealed significant antibacterial actions against *E. coli*. The anti-microbial effect might be due to presence of phenolic compounds that are antiseptic in actions and cause oxidation of bacterial cellular structures, as a result limiting their growth. Medicinal plants contain biologically active compounds that have the property to kill the bacteria or inhibit their growth just like the antibiotics do.[24] Some of the phenolic compounds have antimicrobial properties against certain bacterial pathogens like anti H. pylori activity.[25] In herbal medicine, terpenoids play very important roles in treating different ailments.[26] They are being investigated for their antibacterial[27] and antioxidant activity.[28]

Furthermore, AChE is an enzyme that mostly occurs in the nervous system of the humans and other animals. It is chemically serine protease and is able to break down acetylcholine. As a result, the enzyme stops the cholinergic transmission of impulses in the nervous system.[29] The terpenoids fractions present in the selected plants might be responsible for the anti-enzymatic activity. SAL contains different varieties of terpenoids that possess inhibitory effects against AChE enzyme. An example is the plant *Salvia lavandulaefolia*, which contains terpenoids and essential oils that have the ability to inhibit the AChE enzyme effectively and are used in diseases like Myasthenia gravis and Alzheimer's disease (AD).[30,31]

The methanolic crude extract of SAL significantly inhibited AChE and BChE enzymes**.** Inhibition of AChE and BChE enzymes, which breakdown acetylcholine, are considered as a promising strategy for the treatment of AD. A potential source of AChE and BChE inhibitors is provided by the abundance of plants in nature, and natural products continue to provide useful drugs and templates for the development of other compounds[32].An example of the plant extracts like *Rutagra veolens* L, *Lavandula augustifolia* Miller, and *Salvia lavandu laefolia* which contain terpeniods, alkaloids and essential oils that have the ability to inhibit the BChE, AChE enzymes effectively and areused in diseases like Myasthenia gravis and Alzheimer's disease.[30,31]

Furthermore, SALalso showed a significant anti-inflammatory activity when tested in-vitro by inhibiting Lipoxygenase enzyme which has a similar inflammatory mechanism. The two chief enzymes responsible for process of inflammation are COX-1 and COX-2. The substances which can reduce the actions of these two enzymes can help to stop inflammation.[18,33] SAL has important constituents such as flavonoids, tannins, phenols, alkaloids and carbohydrates that play important roles in treating the inflammation.[34] This elucidates that the crude extract of *Salvia haematodes* has good anti-inflammatory properties and need to be further investigated.

Conclusion

This is elaborated that SAL has considerable anti-oxidant, anti-bacterial, anti lipoxygenase and anti-cholinesterase activities and requires further studies to isolate and characterize the active components.

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