Antimicrobial, Phytotoxic, antithrombotic and anti-hemolysin activities of *Sambucus wightiana* in Swat, Pakistan

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Abstract

Medicinal plants can treat various ailments; hence, the Sambucus wightiana was screened for antibacterial, antifungal, phytotoxic, hemagglutination, antithrombotic and antihemolytic activities. The aerial components of Sambucus wightiana, obtained from Swat, Khyber Pakhtunkhwa, Pakistan, were gathered, identified, and treated. Following the process of methanol extraction, a variety of fractions were acquired. The antibacterial and antifungal activity was evaluated using the agar well diffusion and SDA techniques. The assessment of phytotoxicity was conducted utilizing Lemna minor. The presence of hemagglutination activity against human erythrocytes, antithrombotic activity, and anti-hemolysin activity were assessed. The study offers a comprehensive comprehension of the plant's potential bioactivities. The crude methanolic extract (Cr. MeOH Ext.), n-hexane, and chloroform (CHCl3) showed moderate activity against Staphylococcus aureus, E. coli, S. marcescens, and S. typhi, while the ethyl acetate (EtOAc) and aqueous fractions showed good inhibition. EtOAc and aqueous fractions suppress Alternaria alternata and Fusarium oxysporum, also inhibiting Trichoderma harzianum. Both Cr. MeOH ext and CHCl3 fractions have good and moderate efficacy against Paecilomyces fulvus, F. oxysporum, and Polysphondylium pallidum. Test samples' phytotoxic activity against Lemna minor (L) was concentration-dependent; Cr. MeOH Ext. had good activity, whereas aqueous and EtOAc fractions had moderate activity. The Cr. MeOH ext, n-hexane, EtOAc, and aqueous fractions of the plant had moderate antithrombotic activity, while CHCl3 had low. Erythrocytes of all ABO blood groups did not haemagglutinate. The percent antihemolysin activity against haemolytic E. coli varied by concentration: CHCl3 fraction (71.50), EtOAc (67.59), Cr. MeOH Ext. (66.86), n-hexane (59.50), and water (51.60) at 300. This research underscores the

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therapeutic potential of Sambucus wightiana and contributes to understanding its diverse bioactivities against microbial infections and clot-related concerns. The plant's effectiveness against these health-related issues signifies its possible application as a natural remedy or a source for developing novel therapeutic agents.

Keywords: Sambucus wightiana, Antibacterial, antifungal, phytotoxic, antithrombotic, antihemolysin

Introduction

Around the globe about 60% people of the world population rely on traditional medicine for their primary health care. It has been found that, in developing countries, about 43% of total deaths occurred due to infectious diseases in recent years. According to a report issued by World Health Organization (WHO), fruitful progress has been made in controlling major infectious diseases using traditional medicines (Krishnaswamy, 2008). Many chemically synthesized drugs are used for curing different infectious diseases but over time these microorganisms, which are causing such infections, are getting resistant to the drugs used for inhibiting its growth. In such cases use of alternative modes must be opted and the uses of natural products are among them (Jones et al., 2006). Plants are rich sources of bioactive compounds; steroids, tannins, volatile oils, fixed oils alkaloids, used for controlling the growth of several different pathogenic microorganisms. Since time use of wild plants for nhaveitional and medicinal purposes have become common among various regional groups. Due to fewer side effects and easy availability, the utilization of different medicinal plants is expanding quickly (Hemaiswarya et al., 2008).

In addition to that, there is also a change in the spectrum of pathogens causing different infections in humans. It has been found that it is because of multi-drug resistant pathogens that increased to various diseases (Gould et al., 2010). Among these pathogens *Vancomycin resistant staphylococcus aureus* (*VRSA*) and *Methicillin resistant staphylococcus aureus* (*MRSA*) are of major concern. Worldwide a major group of people are using different plants for medicinal purposes but still less than 10% of the whole medicinal plants have been screened. *Sambucus wightiana* is a shrub belonging to genus Sambucus and family Adoxaceae. Species of this genus is mostly called as elderberry. Plants of this genus are widely distributed in temperate to subtropical region of the world. These species are found in Northern and Southern hemisphere of the globe whereas, in Pakistan these plants are widely distributed in Northern region. Due to its diverse geographical and habitat condition, it

harbors a great wealth of medicinal plants. These elderberries contain 80% of water, 18% carbohydrates and 1% protein making it as a dietary supplements and for curing many diseases; flu and colds (Gould et al., 2010). Because of diuretic and bacteriostatic action, *S. ebulus* L is well known in Roman folk medicine.

Similarly, fully ripened fruits of Sambucus species are rarely used for eating purpose but they are processed in food industries in preparation of jams and jellies. However, *In-vitro* studies suggest that these berries possess anti-carcinogenic, antioxidant, antibacterial and anti-inflammatory activities due to the presence of anthocyanin and other polyphenolics (Gray et al., 2000). *S. wightiana* is employed as a traditional remedy for dermatological ailments. The berries, roots, and leaves have been utilised as laxatives, purgatives, and antimicrobials. Phytochemical research, which is guided by ethnopharmacological expertise, is often considered a suitable method for identifying novel compounds from plants found at higher altitudes (Chashoo et al., 2012). Keeping in view traditional medicinal importance of genus of the selected plant the present study was designed to explore the Cr. MeOH Ext. and various organic fractions (*n*-hexane, $CHCl_3$, EtOAc and aqueous) for antibacterial, antifungal, phytotoxic, hemagglutination, antithrombotic and anti-hemolytic activities per standard procedures.

Material and Methods

Collection, Extraction and Fractionation

The plant's aboveground components were gathered from Swat, Khyber Pakhtunkhwa, Pakistan and were authenticated by Dr. Lal Badshah, a botany expert from the University of Peshawar. The plant material underwent shade drying and was thereafter chopped and ground into a fine powder. Subsequently, the powdered substance was immersed in methanol for a duration of 15 days at ambient temperature. The part soluble in methanol was filtered after a period of 15 days and concentrated at a temperature of 42 degrees Celsius using a rotary evaporator. Several fractions were obtained from Cr. MeOH Ext. by suspending it in 500ml of distilled water and separating it into n-hexane (190g), CHCl3 (225g), EtOAc (210g), and an aqueous fraction (290g) through partitioning with n-hexane (3x500ml), CHCl3 (3x500ml), and EtOAc (3x500ml).

Antibacterial Activity

This activity of test samples was determined by agar well diffusion method (Ahmad et al., 2009). Uniform lawns consisting of various bacterial pathogens, including *Salmonella typhi, S. aureus,*

Pseudomonas aeruginosa, *E. coli*, and *Serratia marcescens* were created on sterile nutrient agar plates. Subsequently, using a 6mm borer, holes were drilled into the plates. 100µl of the test samples were added to the corresponding wells from the stock solution, which contained a concentration of 3mg/ml of Dimethyl Sulfoxide (DMSO) at a maximum of 1%. The plates were placed in an incubator at a temperature of 37 degrees Celsius for a duration of 24 hours. The diameter of the zone of inhibition (measured in millimetres) was recorded. The percentage of inhibitory impact was assessed by comparing it with the standard.

Antifungal Activity

To assess antifungal activity, a stock solution of test samples was created by dissolving 24mg/ml of DMSO. The aseptic Sabouraud Dextrose Agar (SDA) plates were utilised to cultivate the following fungal species: F. oxysporum, A. flavus, Rhizopus stolonifer, A. alternata, T. harzianum, P. fulvus, and Penicillium pallidum. In activity 4, 4 millilitres of SDA medium were prepared in test tubes. After autoclaving, when the temperature reached approximately 45 degrees Celsius, 66.6 microliters of the test samples were added to their corresponding test tubes. The tubes were positioned at an angle to create a slant and then inoculated with 7-day-old test fungus. The negative control, DMSO (≤1%), and the positive control, Miconazole, were utilised (Azam et al., 2016).

Phytotoxic Activity

For phytotoxic activity of the test samples, *Lemna minor* was used as reported (Hussain et al., 2010). The test samples (20g) were dissolved in methanol and E medium was prepared as per instruction. The test samples were introduced into respective flask at concentration of 10, 100 and 1000µg/ml from stock solution and MeOH was allowed to evaporate. After evaporation, 20ml of E. media and healthy *L. minor* plants were introduced into respective flasks and incubated at 28±1°C for 7 days. Paraquat (0.015µg/ml) was used as standard and three flasks containing *L. minor* and E-media only as negative control. After 7 days results were recorded.

Hemagglutination Activity

The Hemagglutination activity of the test samples was performed against human erythrocytes of ABO blood groups (Lau et al., 2009). Stock solution (1mg/ml) of the test samples were prepared and were serially diluted (1:2, 1:4, 1:8 and 1:16). The blood (5cc) was collected and centrifuged to obtain the erythrocytes. In phosphate buffer (pH 7.4), 2%

erythrocyte suspension was prepared for all blood samples. In the next step, 1 ml sample was taken per dilution and was mixed with 1 ml of "erythrocyte suspension" in a test tube. The test tubes were incubated at 37°C for 30 minutes and the results were recorded. An irregular and smooth button formation indicates positive and negative results respectively.

Antithrombotic Activity

To determine antithrombotic activity of *S. wightiana*, fresh blood (2.5ml) was taken from healthy volunteers and was equally distributed in 5 different pre-weighed (W_o) sterile micro-centrifuge tubes. The tubes were incubated at 37°C for 40-50 minutes and after clot formation serum was removed from each tube very cautiously and was again weighed (W1) to determine the clot weight. Then 100µl of each extract from the stock solution (10mg/ml of water) was added to already weigh centrifuge tubes and incubated at 37°C for 90 minutes. After incubation, fluid released in centrifuge tubes was removed and were again weighed (W2) observing the difference in weight (W2). Streptokinase and distilled water were utilized as positive and negative control, respectively (Bojić et al., 2019; Martinichen-Herrero et al., 2005). The difference between weight obtained before and after clot lysis was express in percent clot lysis.

% Degradation = (Weight of clot degraded / Net Weight of clot) x 100
Weight of centrifuge tube = W_o,
Weight of tube + Weight of clot before lysis = W_1
Weight of tube + Weight of clot after lysis = W_2,
Weight of clot degrade = $W_1 - W_2$

Anti-hemolysin Activity

Sambucus wightiana was screened to check its potential to inhibit the activity of hemolysin produced by *E. coli*. To perform the experiment, solution (stiock) of test samples was prepared by dissolving 1mg/ml of saline buffer. The hemolysin producing *E. coli* was inoculated to 1ml of nutrient broth and incubated at 37°C for 24 hours. After incubation the broth was centrifuged resulting in two layers, pellet, and supernatant. The supernatant contains the hemolysin was removed for further experimental work. Red Blood Cells (RBCs) suspension (2%) was prepared in phosphate buffer (pH 7.4). About 0.5ml of supernatant and 1ml of the stock solution was mixed with 1ml of RBCs suspension and incubated for 2-3 hours at 37°C followed by centrifugation at 8000 rpm for 3 minutes. Clear supernatant indicates hemolysin inhibition whereas red color

showed hemolyzed RBCs and the intensity were observed spectrophotometrically at 540nm (Vane & Botting, 2003).

Results

Antibacterial Activity

Antibiotic resistance is a major medical issue. Most serious disease-causing bacteria are difficult to control because they can resist drugs (Nyiligira et al., 2008). Plants have various secondary metabolites that can limit pathogen growth. Figure 1 shows *S. wightiana* test samples evaluated for antibacterial activity. The Cr. MeOH Ext. inhibited *S. aureus* (46.1%) but not *P.aeruginosa* (38.5%), *E. coli* (34.7%), *S. marcescens, or S. typhi* (30%). The test sample was inert against *S. aureus* and S. typhi, while the n-hexane fraction inhibited *E. coli, S. marcescens, and P. aeruginosa* by 56.52, 56.52, and 53.8% CHCl3 fraction of plant was effective with intermediate results against, *E. coli (52.1%), S. marcescens (57.1%) and S. typhi (45%),* low against *S. aureus* (30.7%) and no inhibition against P. aeruginosa. The EtOAc fraction inhibited S. marcescens (66.6%) and E. coli (47-82%). Low activity against *S. aureus* (26.9%), inert against S. typhi and P. aeruginosa. Aqueous fraction of plant had good activity against S. typhi (60%) and modest activity against *P. aeruginosa* (27%), inert against remainder of test bacterial pathogens.

Antifungal Activity

The results of the antifungal inhibitory test against test fungus species are shown in figure 2. The aqueous component of the plant reduced F. oxysporum linear growth (90%) and did well against *P. fulvus (75%) and T. harzianum* (70%). Other test fungi: *R. stolonifer, A. alternata, P. pallidum, and A. flavus* showed 54.50, 50, 40, and 35% inhibition. EtOAc had 80 percent activity against *A. alternata*, 75 percent against F. oxysporum, 70 percent against T. harzianum, and 70 percent against P. fulvus. This fraction has modest activity against P. pallidum (27.3), R. stolonifer (27%), and A. flavus (22.22%). The Cr. MeOH Ext. inhibited, P. pallidum (46%), F. oxysporum (50.0%) and P. fulvus (64%). Low activity against R. stolonifer (20%), A. alternata (17%), and A. flavus (9%), inert against T. harzianum. The CHCl3 fraction has 42% activity against F. oxysporum, P. fulvus, and P. pallidum. Low activity of 19, 17, 16, and 2.50% was detected against A. flavus, T. harzianum, R. stolonifer, and A. alternata. The n-hexane fraction of the plant has moderate activity against T. harzianum (58.33%) but poor activity against P. fulvus (36.63%), A. flavus (25%), F. oxysporum (20%), A. alternata (17%), R. stolonifer, and P. pallidum (8.33%).

Phytotoxic Activity

It has been reported that herbicides of plants origin are environment friendly. In search of finding the phytotoxic ability of various components the *L. minor* is used as a model (Hussain et al., 2010). of the selected plant are given in figure (3). Cr. MeOH Ext. had 60% phytotoxic activity at 1000µg/ml, but low activity at 100 (30%) and 10µg/ml (20%). A considerable phytotoxic effect was detected at 1000 and 100µg/ml for the aqueous fraction (50 and 40%), but minimal activity at 10µg/ml. "N-hexane, CHCl3, and EtOAc fractions of the plant exhibited low activity (10-30%) at all test concentrations as shown in figure 3."

Haemagglutination Activity

The selected plant was also screened for the presence of phytolectins as plants can serve as an abundant and cheap source of agglutinins. The test samples (Cr. MeOH Ext. and various organic fractions) were screened at various dilutions (1:2, 1:4, 1:8 and 1:16) for determination of haemagglutination potential. As shown in table 1, all the test samples at all dilutions showed no haemagglutination activity.

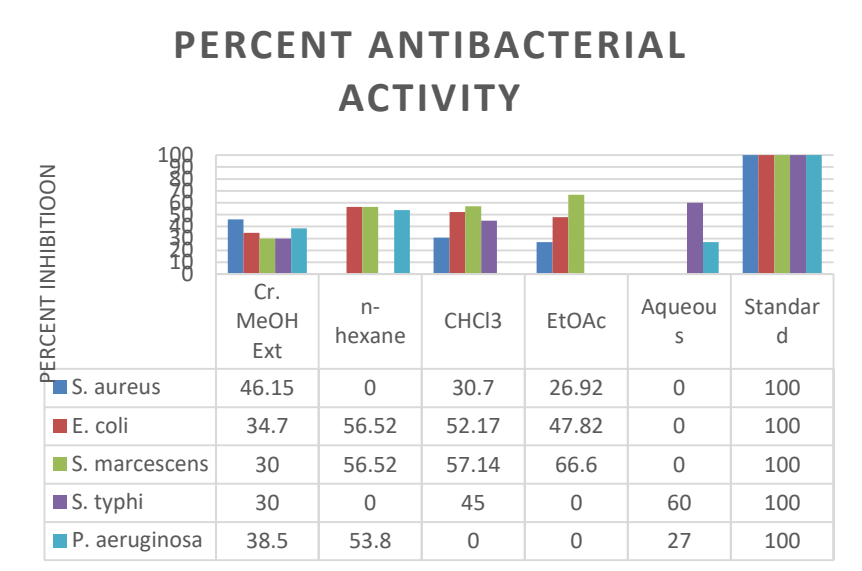
Antithrombotic Activity

The results of the antithrombotic potential of Cr. MeOH Ext. and various fractions of *S. wightiana* are presented in figure 4. The EtOAc, aqueous, Cr. MeOH Ext. and *n*-hexane fraction presented a significant antithrombotic potential with percent clot lysis of 52.28, 48.21, 47.98 and 42.32, respectively. The $CHCl_3$ fraction of the selected plant, on the other hand, showed low antithrombotic activity (6.94%). In the current study, the streptokinase served as "positive control" with 68.43% activity while the "negative control (water)" showed no clot lysis.

Antihemolysin Activity

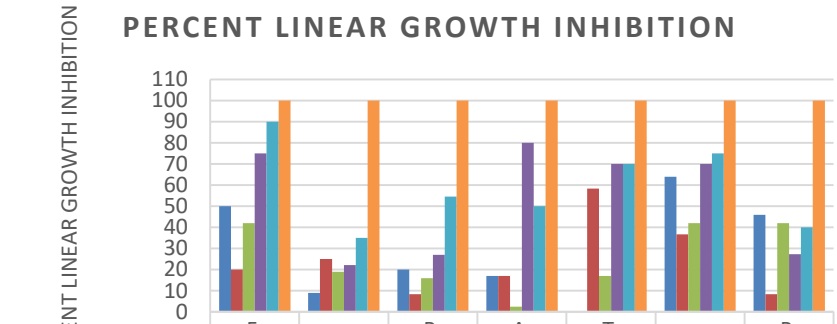
The hemolysin inhibitory potential of the selected plant was observed against the hemolysin producing *E. coli*. The results of the activity at various concentrations (50, 100 and 300µl) are presented in figure 5. The Crd. MeOH Ext possesses good antihemolysin effect (66.86%) at 300µl, moderate (45.5%) at 100µl and low potential (31.5%) was observed at 50µl. The percent antihemolysin effect of the *n*-hexane fraction at 300, 100 and 50µl was 59.5 (good activity), 38.54 and 27.51 (low potential), respectively. The percent antihemolysin potential of the $CHCI_3$ fraction of the selected plant in the current study was good at 300µl (71.5), moderate at 100µl (53.25) and low at 50µl (32.25). The EtOAc

fraction presented good and moderate inhibitory potential (67.59, 45.75%), respectively at higher concentrations while it showed low activity (29.67%) at lower concentration. The percent hemolysin inhibitory potential of the aqueous fraction was moderate at 300µl (51.6), low at 100µl (30.42) and low at 50µl (19.4).



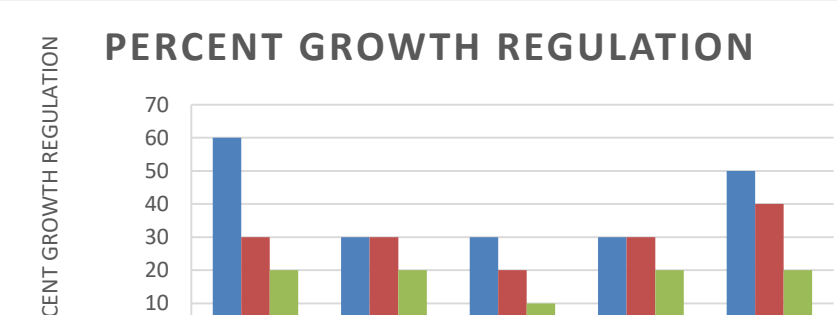
	Cr. MeOH Ext	n-hexane	CHCl3	EtOAc	Aqueous	Standard
S. aureus	46.15	0	30.7	26.92	0	100
E. coli	34.7	56.52	52.17	47.82	0	100
S. marcescens	30	56.52	57.14	66.6	0	100
S. typhi	30	0	45	0	60	100
P. aeruginosa	38.5	53.8	0	0	27	100

Figure 1: Percent antibacterial activity of Sambucus wightiana.



	F. oxysporum	A. flavus	R. stolonifer	A. alternata	T. harzianum	P. fulvus	P. pallidum
Cr. MeOH Ext	50	9	20	17	0	64	46
n-hexane	20	25	8.33	17	58.3	36.63	8.33
CHCl3	42	19	16	2.5	17	42	42
EtOAc	75	22.22	27	80	70	70	27.3
Aqueous	90	35	54.5	50	70	75	40
Standard	100	100	100	100	100	100	100

Figure 2: Percent linear growth inhibition of Sambucus wightiana



	Cr. MeOH Ext	n-hexane	CHCl3	EtOAc	Aqueous
1000 µg/ml	60	30	30	30	50
100µg/ml	30	30	20	30	40
10µg/ml	20	20	10	20	20

Figure 3: Percent growth regulation of Sambucus wightiana against Lemna minor

Discussion

Recently much focus has been given to traditionally used medicinal plants because of its low toxicity and fewer side effects which is mostly associated with synthetic drugs (Al-Zoreky, 2009). The present study was conducted to explore the pharmacological potentials of *S.*

Table 1
Haemagglutination activity of Sambucus wightiana

Blood Group	**All ABO Blood Groups**			
Test Dilutions.	**1:2**	**1:4**	**1:8**	**1:16**
Aqueous.	--	--	--	--
Ethyl Acetate.	--	--	--	--
Chloroform.	--	--	--	--
n-hexane.	--	--	--	--
Cr. MeOH. Ext.	--	--	--	--

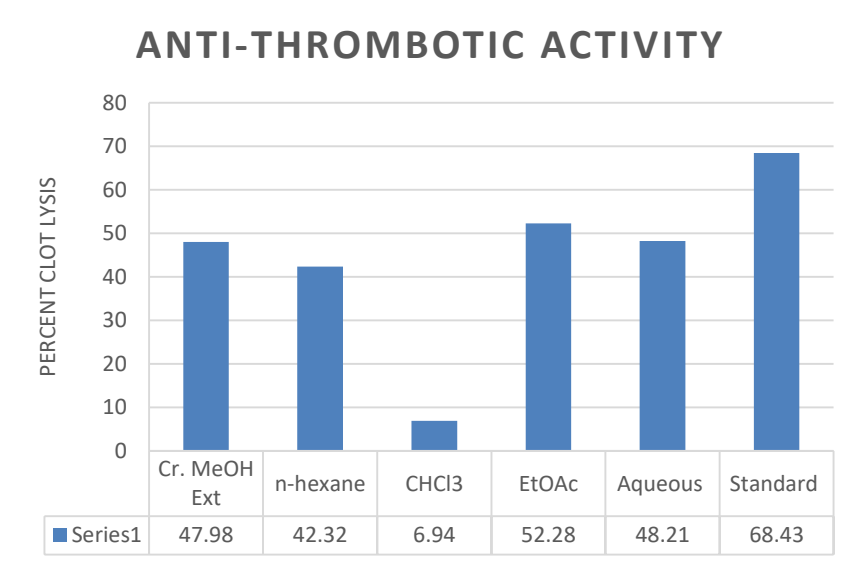


Figure 4: Anti-thrombotic activity of Sambucus wightiana

wightiana and the results showed that it contains certain bioactive constituents that can control the growth of various mentioned pathogens. *Sambucus ebulus* and *Urtica dioica* were screened against Methicillin Resistant *S. aureus* (MRSA) clinical isolates and *S. ebulus* showed a zone of inhibition was 14-22mm, while that of *U. dioica* was 10-21mm (Ebrahimzadeh et al., 2009). Similarly, *S. nigra* flower and elder berry was screened against MRSA and it showed good results against it. The Cr. MeOH Ext. of this plant also presented good wound healing activity

(Veberic et al., 2009). In the current study the selected plant also showed good and moderate inhibitory effect against the selected bacterial pathogens. Vascular wilt, rice sheath blight, fruit rot and many more are plant fungal diseases. Different chemical fungicides are very effective in controlling various diseases, but they cannot be persistently used because of their health and environmental hazards.

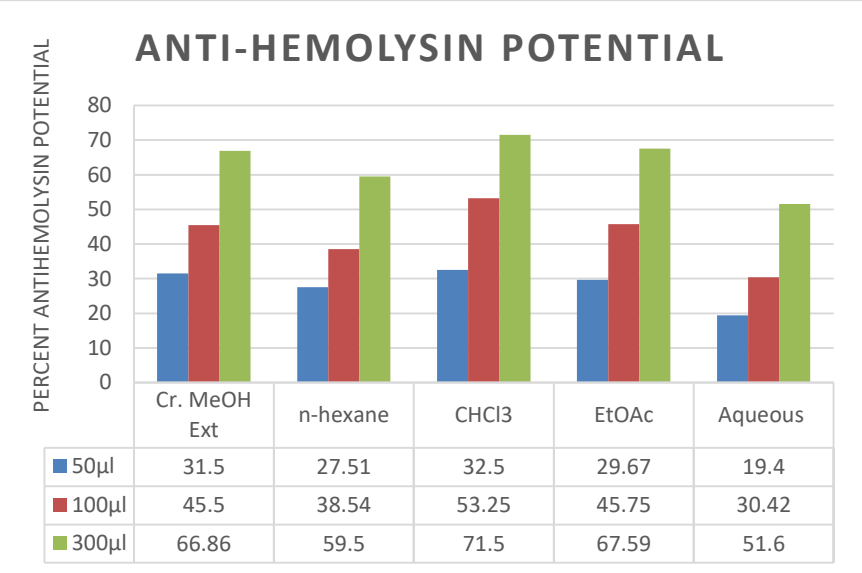


Figure 5: Antihemolysin potential of Sambucus wightiana

There is a need for replacing synthetic chemicals with alternatives that should be effective and not risky to humans and environment (Ahmad et al., 2009). *Sambucus wightiana* possess the ability of inhibiting the growth of fungi i.e. the aqueous fraction presented a significant activity against *F. oxysporum*. Similarly antifungal activity of some plant's species; *Solanum* sp. and *Juglans* sp. extracts, against *Microsporum canis* and *Trichophyton mentagrophytes* have also been reported. Various anticoagulant, antiplatelet and antithrombotic therapies, like aspirin (derived from willow leaves), have been derived from plants. Similarly, warfarin, an anticoagulant was derived from sweet clover plants (Vane & Botting, 2003). Curcuma oil of *curcuma longa* has the antithrombotic potential (Saputri & Avatara, 2018). The current study is evidence that *S. wightiana* has antithrombotic effect. In the current study it was observed that the selected plant species has antihemolysin and phytotoxic potential but lacks phytoagglutinins.

The current investigation on *Sambucus wightiana* substantiates its pharmacological properties by drawing certain similarities with previous studies. The novel investigation distinguishes itself from prior ones through many means. This study demonstrates the antimicrobial efficacy of *S. wightiana* against MRSA, like previous examinations conducted on *S. ebulus, S. nigra,* and Urtica dioica. Elderberry species possess inhibitory qualities that enable them to combat antibiotic-resistant bacteria in a similar manner (Álvarez-Martínez et al., 2020). The antifungal activity of *S. wightiana* is consistent with the growing trend of using plant-based fungicides. Other studies conducted on different plant species with a focus on fungal pathogens demonstrate a common interest in using botanical methods to cure agricultural diseases (Kunwar et al., 2010).

The antithrombotic effects of *S. wightiana* are in line with the long-established use of plant-derived anticoagulants. This study provides evidence for the role of plants as reservoirs of bioactive compounds that have cardiovascular effects (Sripathi et al., 2017). This research provides a comprehensive analysis of the inhibitory effects of *S. wightiana* on specific bacterial illnesses. This specialisation enhances our understanding of the antibacterial actions exhibited by the Sambucus genus.

The study discovered the phytotoxic capabilities of *S. wightiana*, indicating its ecological role in plant defence. The absence of phytoagglutinins sets *S. wightiana* apart from other plants, highlighting its distinctive biochemical profile. The study implies that the methanol extract of S. wightiana has potential in promoting wound healing, but it is not explicitly stated in the comparison. This discovery introduces a therapeutic aspect that has not been addressed in the existing literature.

Conclusion

The plant displayed promising effects in inhibiting the growth of certain pathogens. Specifically, the methanolic extract and different fractions showed varied yet considerable activity against bacterial strains like Staphylococcus aureus and Escherichia coli, as well as fungal species including Alternaria alternata and Fusarium oxysporum. Additionally, it demonstrated significant antithrombotic potential and the ability to hinder hemolysin activity produced by Escherichia coli. However, it didn't exhibit phytoagglutinin activity. This research underscores the therapeutic potential of Sambucus wightiana and contributes to understanding its diverse bioactivities against microbial infections and clot-related concerns. The plant's effectiveness against these health-related issues signifies its possible application as a natural remedy or a source for developing novel therapeutic agents.

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