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RESEARCH ARTICLE

COMPARATIVE ANTI-*TRICHOMONAS VAGINALIS* ACTIVITY EVALUATION OF *SAMBUCUS NIGRA* L. FLOWERS AND FRUITS EXTRACTS

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Abstract

Background: *Sambucus nigra* or also known as Black elderberry or European elderberry is been used for the teatment and prevention of various diseases. The plant posseses health beneficial effects such as antimicrobial, antioxidant, antiulcerogenic, antidepressant, antidiabetic, antiviral and antiinflammatory. However, there is limited report on the antitrichomoniasis activity of *S. nigra*.
Objectives: The study aimed to comparatively analyze the *in vitro* trichomonicidal effects of various extracts prepared from both fruits and flowers of *S. nigra*. Half maximal inhibitory concentration (IC_{50}) and minimum parasiticide (MPC) values of the extracts against metronidazole-resistant *Trichomonas vaginalis* 50143 strain were determined by the liquid microdilution method using 96-well microplates.
Results: After 48 hrs incubation with parasites, the highest activities were observed for 70% hydroethanolic extract, chloroform extract and methanol extract of fruits with IC_{50} values of 513, 531 and 566 µg/ml, respectively. Fruit extracts exhibited higher activity than flower extracts, and no activity was observed in methanol extract of flowers.
Conclusion: This study is the first comparative analysis of the anti-*Trichomonas vaginalis* activity of elderberry extracts. This study will serve as a reference source in isolating the components of elderberry fruits with antiparasitic activity.
Keywords: Metronidazole, plant extracts, *Sambucus nigra*, *Trichomonas vaginalis*, trophozoites.

INTRODUCTION

Sambucus nigra L., distributed in Europe, North Africa, West and Central Asia and North America, is one of the two species growing wildly in Turkey. This perennial shrub or 8-10 m tall tree with purplish fruit and creamy white flowers belongs to Adoxaaceae family[1-3]. *S. nigra* is also known as Black elderberry or European elderberry and believed to have been used since ancient times for the treatment and prevention of various diseases[4,5]. In traditional Chinese medicine *S. ebulus* or known as dwarf elder is widely used in bone and joint disorders[6]. In Europe and African countries, *Sambucus* species are traditionally used in the treatment of respiratory diseases such as asthma and bronchitis, throats, sinusitis, herpes, neuralgia, epilepsy and for dental problems for bronchitis and stomach ache[7,8]. In Turkey, in addition to external use for the treatment of rheumatism and wounds, the decoction or infusion prepared from flowers and fruits of *S. nigra* are used as diuretic, diaphoretic, immune system booster and protective against cancer. The fruits are also used in the carpet and textile industry due to its colorful structure[9-12].

Numerous pharmacological activities have been conducted on the fruits of *S. nigra* and proven to posses health beneficials such as antimicrobial, antioxidant, antiulcerogenic, anti-depressant, antidiabetic, antiviral and anti-inflammatory[8,13]. Due to various health benefits, the elderberry fruits are also included in the composition of jam, pie, ice cream, yoghurt, wine, tea and fruit juices[6].

Trichomoniasis is one of the most common sexually transmitted diseases caused by *T. vaginalis* whose annual average prevalence is around 156 million in the world[14]. *Trichomonas* manifests itself with a number of symptoms, and different symptoms occur in men and women. This disease is also reported to be the causative agent of cervical cancer, pelvic inflammation and infertility[15]. Metronidazole is the most effective drug used in the treatment of trichomoniasis. Unfortunately, besides drug resistance, side effects

such as gastrointestinal disorders, taste problems, nausea, vomiting, vertigo, neutropenia, skin reactions, cloudy urine and fatigue are reported for metronidazole[16-19]. Due to serious side effects of 5-nitroimidazole derivatives, a more accesible source of drugs with fewer side effects are still being investigated from natural sources[20].

In a study conducted in Iran, plants registered in international databases were searched for their *in vitro* anti-*Trichomonas* activity. Garlic, lavender, eucalyptus, geranium, onion, yarrow, wormwood which are traditionally used for antiparasitic activities were found to be effective against *T. vaginalis*[21]. In other investigations conducted to find alternative sources for the treatment of trichomoniasis, rhubarb, turmeric and fennel were found to inhibit the growth and reproduction of parasites[22,23]. In a recent study, Niknam *et al.*, reported the anti-*T. vaginalis* effect of methanolic extract prepared from *S. nigra* fruits from nothern Iran[24].

To the best of our knowledge this is the first comparative research in scientific literature on the anti-*Trichomonas* activity of elderberry fruits and flowers. In this study, the extracts were prepared from fruits and flowers of *Sambucus* by using solvents with different polarities and are analyzed for their *in vitro* antiparasitic activity against *T. vaginalis*. While there have been recent studies on the antioxidant, antimicrobial, cytotoxic and antiviral activities of *S. nigra*[6,8], it is surprising that only one study has been conducted on the activiy of *S. nigra* fruits against the common *T. vaginalis* parasite[24]. This *in vitro* anti-*T. vaginalis* activity research will provide a basis for future *in vivo* trichomonocidal activity studies on *S. nigra*.

MATERIALS AND METHODS

Plant materials and preparation of extracts

S. nigra flowers and fruits were collected in June and August 2023, respectively. The plant species was authenticated by pharmacist Muammer Şen from Konya. The plant materials were dried at room temperature. The dried fruits and flowers were purchased from Pharmacist Muammer Şen, whose the owner of Temmuz Organic Farm, Selçuklu, Konya, Turkey. An amount of 5 g of fruits and flowers were grounded and macerated with solvent (25% hydroethanolic solution, 70% hydroethanolic solution, methanol and chloroform) with a plant/solvent ratio of 1:10 at room temperature under stirring for 48 hrs. The extracts were filtered through Whatman no.1 paper and solvent was evaporated to dryness by rotary evaporator under reduced pressure. The extracts were lyophilized and stored at -20°C until analysis[25].

Preparation of Tyrypticase Yeast Extract Maltose (TYM) Medium

Metronidazole resistant reference strain *T. vaginalis* ATCC 50143 was obtained from Celal Bayar University, Faculty of Medicine, Parasitology Bank, Manisa. In order to revive *T. vaginalis* parasites and to bring to logarithmic phase, tyrpticase yeast, maltose (TYM) medium was used. TYM medium is mainly composed of 20 g trypticase, 5 g maltose, 10 g yeast extract, 0.2 g L-ascorbic acid, 1 g L-cysteine, 0.5 g agar and 0.8 g pH regulators (K_2HPO_4-KH_2PO_4). Materials other than agar were weighed in the required amounts and dissolved in 900 ml distilled water using magnetic stirrer. The pH was adjusted to 6, followed by addition of agar and the mixture was sterilized for 20 minutes at 121ºC under 15 psi. At the end of sterilization process, TYM medium was divided into 4 ml screw cap glass tubes. To make a total volume of 5 ml, before use, 100 IU/ml streptomycin, 100 IU/ml penicillin and commercially obtained inactive horse serum were added to TYM medium[26,27].

Revitalizing the parasites and bring into logarithmic phase

T. vaginalis strain was quickly thawed in a 37ºC water bath within 1.5-2 minutes and then centrifuged and washed twice with fresh medium to remove remaining DMSO. After washing, the parasites accumulated at the bottom were added to TYM medium with 16% horse serum and incubated in the oven at 37ºC.The growth of parasites was observed under a microscope. Then, the strains were then transferred to fresh medium to ensure average adaptation. Parasites that receive sufficient and necessary substances and pass the latent phase are expressed as production by multiplying their numbers in a generation period specific to their species is defined as logarithmic phase. Strains entering logarithmic phase were transferred to cell flask and incubated for the growth of parasites in high volumes[27,28].

Investigation of the antitrichomonial activity of extracts

Half maximal inhibitory concentration (IC_{50}) and minimum parasiticide concentration (MPC) values of the extracts against metronidazole-resistant *T. vaginalis* 50143 strain were determined *in vitro* by the liquid microdilution method in 96-well microplates[28]. After the trophozoites entered the logarithmic phase, they were counted on the Thoma chamber using trypan blue stain and their viability was determined. In the experiments, trophozoites with a survival rate of over 95% were used and the amount of parasites was adjusted to $5x10^3$ parasites/ml using a Thoma chamber. $5x10^3$ parasites/ml *T. vaginalis* trophozoites were added to each well except for the negative control and the microplates were incubated at 37°C for 48 hrs. At the end of incubation, the motility of *T. vaginalis* trophozoites was evaluated by direct examination under an inverted microscope, and parasite viability was determined using trypan blue. For growth control (positive control), two wells containing *T. vaginalis* without tested extracts were used, and for contamination control (negative control), two wells containing the extracts without *T. vaginalis* were used. Then, the plates were incubated at 37°C under aerobic conditions. At the 24th and 48th hrs of incubation, the motility of *T. vaginalis* trophozoites was evaluated under an inverted microscope, and their viability was evaluated on a Thoma counting chamber using trypan blue stain. The extract concentration in the last well, where approximately half of the *T. vaginalis* trophozoites were determined to be alive using trypan

blue stain, was accepted as the IC_{50} value and the MPC value was determined by taking trophozoites from the wells containing immobile parasites, passage them into a new TYM medium, and checking for growth at the 24th and 48th hrs[29].

Statistical analysis

The results of three parallel studies carried out at two different times were averaged and the viability percentages corresponding to all concentrations in which two-fold serial dilutions were analyzed with the Graphpad program and IC_{50} and MPC values were expressed as µg/ml of the extracts.

RESULTS AND DISCUSSION

In this study, the anti-*T. vaginalis* activity of *S. nigra* fruits and flowers extracts prepared with solvents of different polarities was comparatively analyzed. The parasite viability percentages of the fruit and flower extracts at 24 and 48 hrs are demonstrated in Table 1, Table 2, and Table 3, Table 4 respectively. The extracts of *S. nigra* reduced the number of live *T. vaginalis* parasites in a time and concentration dependent manner. The fruit extracts exhibited higher trichomonocidal activity then the flower extracts. As shown in Table 5, after 24 hrs of incubation the chloroform and 70% hydroethanolic extracts of fruits showed the highest inhibition of parasites with IC_{50} values of 1136 and 1138 µg/ml respectively.

Table 1: Viability percentages of *T. vaginalis* trophozoites at 24 hrs in the presence of *S. nigra* fruits extracts.

Concentration (µg/ml)	70% hydroethanolic extract	25% hydroethanolic extract	Chlorofom extract	Methanol extract
25000	0.00±0.00	0.00±0.00	00.00±0.00	8.00±1.73
12500	0.00±0.00	40.33±0.57	0.00±0.00	50.00±0.00
6250	0.00±0.00	50.00±0.00	0.00±0.00	79.00±3.46
3125	13.33±2.88	63.33±0.57	10.66±2.30	92.33±2.88
1562.5	50.00±0.00	77.33±2.88	50.00±0.00	100.00±0.00
781.25	56.00±1.73	87.33±1.15	60.00±3.46	100.00±0.00
390.625	79.33±0.57	95.66±1.15	73.33±2.88	100.00±0.00
195.3125	95.66±1.15	100.00±0.001	93.66±23	100.00±0.00
97.65625	100±0.00	100±0.001	100±0.001	100.00±0.001

Table 2: Viability percentages of *T. vaginalis* trophozoites at 24 hrs in the presence of *S. nigra* flowers extracts.

Concentration (µg/ml)	70% hydroethanolic extract	25% hydroethanolic extract	Chlorofom extract	Methanol extract
25000	0.00±0.00	0.00±0.00	8.00±1.73	100±0.00
12500	0.00±0.00	0.00±0.00	50.00±0.00	100±0.00
6250	7.33±0.57	7.00±1.73	79.00±3.46	100±0.00
3125	50.00±0.00	50.00±0.00	92.33±2.88	100±0.00
1562.5	59.33±0.57	55.66±2.88	100.00±0.00	100±0.00
781.25	77.33±2.88	74.33±2.88	100.00±0.00	100±0.00
390.625	86.33±2.88	89.00±1.73	100.00±0.00	100±0.00
195.3125	99.00±1.73	100.00±0.00	100.00±0.00	100±0.00
97.65625	100±0.00	100.00±0.00	100.00±0.00	100±0.00

S. nigra fruit methanolic extracts' IC_{50} were calculated as 1373 and 566 µg/ml for 24 hrs and 48 hrs respectively whereas no activity was observed at 48 hrs for the methanolic extracts of flowers even at concentration 25000 µg/ml. Among the flower extracts, the highest activity was observed in the chloroform extract with IC_{50} value at 605 µg/ml. The 70% hydroethanolic extract of fruits exhibited the highest trichomonocidal activity with 513 µg/ml IC_{50}, while it was 8 µg/ml for metronidazole. In a study conducted on trichomonocidal activities of herbs used in Korea, the methanolic extracts of Torilidis fructus, Sophorae radix and Agrimonae herba were reported to show complete trichomonocidal activity at the concentration of 400 g/ml where as metronidazole used as positive control showed 0.94% viability at the concentration of 5 g/ml[33].

Table 3: Viability percentages of *T. vaginalis* trophozoites at 48 hrs in the presence of *S. nigra* fruits extracts.

Concentration (µg/ml)	70% hydroethanolic extract	25% hydroethanolic extract	Chlorofom extract	Methanol extract
25000	0.00±0.00	0.00±0.00	00.00±0.00	00.00±0.00
12500	0.00±0.00	0.00±0.00	0.00±0.00	00.00±0.00
6250	0.00±0.00	0.00±0.00	0.00±0.00	00.00±0.00
3125	0.00±0.00	8.66±0.57	0.00±0.00	00.00±0.00
1562.5	10.66±0.57	35.33±1.15	7.33±0.57	5.33±0.57
781.25	36.66±2.30	50.00±0.00	32.66±2.88	30.33±2.30
390.625	50.00±0.00	63.00±1.73	50.00±0.00	50.00±0.00
195.3125	74.66±0.57	77.00±1.73	60.00±1.73	66.66±5.77
97.65625	85.66±4.04	89.66±1.15	80±1.73	77.00±1.73

Table 4: Viability percentages of *T. vaginalis* trophozoites at 48 hrs in the presence of *S. nigra* flowers extracts.

Concentration (µg/ml)	70% hydroethanolic extract	25% hydroethanolic extract	Chlorofom extract	Methanol extract
25000	0.00±0.00	0.00±0.00	0.00±0.00	100±0.00
12500	0.00±0.00	0.00±0.00	7.33±1.15	100±0.00
6250	0.00±0.00	0.00±0.00	30.66±1.15	100±0.00
3125	5.66±0.57	7.33±1.15	50.00±0.00	100±0.00
1562.5	31.00±5.19	30.00±1.73	57.66±2.30	100±0.00
781.25	50.00±0.00	50.00±0.00	70.00±1.73	100±0.00
390.625	62.33±1.15	66.00±3.46	87.33±2.88	100±0.00
195.3125	78.66±0.57	76.00±1.73	97.00±1.73	100±0.00
97.65625	87.66±2.30	81.00±1.73	100.00±0.00	100±0.00

Table 5: The comparative *in vitro* activity of *S. nigra* fruit and flowers extracts on *T. vaginalis.*

Extracts	24. hrs (µg/ml)		48. hrs (µg/ml)	
	IC_{50}	MPC	IC_{50}	MPC
70% MM	1138	3125	513	1562
25% MM	8819	12500	886	3125
CMM	1136	3125	531	1562
MMM	1373	3125	566	1562
25% MC	2388	6250	1046	3125
70% MC	2611	6250	924	3125
CMC	2103	6250	605	2500
MMC	>12500	>12500	>12500	>12500
Metronidazole	14	66	8	33

MPC: Minimum Parasiticide Concentration; 70%:70% hydroethanolic extract; 25%: 25% hydroethanolic extract
MM: extract of fruits; MC: extract of flowers. CMM: chloroform extract of fruits. CMC: chloroform extract of flowers
MMM: methanol extracts of fruits; MMC: methanol extracts of flowers

In another survey on the anti-*T. vaginalis* activitiy, hydroalcoholic extracts of *Eugenia caryophyllata*, *Camellia sinensis* and *Terminalia chebula* were reported to have the best anti-trichomonal activity with IC_{50} values of 1.21, 1.62 and 1.66 mg/ml, respectively[34]. In the present study, the 70% hydroethanolic fruits extract which had the highest anti-*T. vaginalis* activity among the investigated fruit and flower extracts, inhibited the 50% of parasites at the concentration of 0.513 mg/ml which is much lower concentration than those extracts which were reported to have significant activity. In a previous report, methanolic extract of *S. nigra* fruits were evaluated for *in vitro* anti-*Trichomonas* activity at concentrations ranging from 100-800 µg/ml and at 800 µg/ml the extract was reported to have 100% efficacy after 48 hrs[24]. In contrast, in this study fruit methanolic extract inhibited the 50% of parasites at 566 µg/ml at 48 hrs. 95% parasite inhibition was observed in methanol extract at 1562.6 µg/ml. Obtained results showed that *S. nigra* has acceptable efficacy *in vitro* and according to minimum parasiticide concentrations fruit extracts were significantly better than flower extracts.

However *in vivo* trichomonocidal activity search is required for further studies. Among the phytochemicals in *S. nigra*, flavonoids, anthocyanins, phenolic acids, cyanogenic glycoside and vitamin A and vitamin C have been reported[8]. Research has been conducted mostly on the phenolic components of the elderberry plant[30-32], but as observed in this study, chloroform extract, which is known to contain more lipophilic compounds, also showed strong activity. In addition to hydroethanolic and methanolic extracts of fruits, the chloroform extracts of fruits and flowers are also worth for investigating antitrichomonocidal candidates.

CONCLUSIONS

There are limited studies on the activity of elderberry against trichomoniasis. This study is the first comparative analysis of the anti-*T. vaginalis* activity of *S. nigra* extracts. This study will serve as a reference source in isolating the components of elderberry fruits with antiparasitic activity. Anti-*T. vaginalis* bioactivity guided assay is planned for further studies for the isolation of active constituents of fruits and flowers of *S. nigra.*

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AUTHOR'S CONTRIBUTION

Erdemir E and Çavus I: investigation, data curation, writing and editing. **Kayalar H:** plant material supply, methodology. **Kayalar H and Ozbilgin A**: supervision Final manuscript was read and approved by all authors.

DATA AVAILABILITY

The data will be available to anyone upon request from the corresponding author.

CONFLICT OF INTEREST

None to declare.

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