

Anti-Toxoplasma activities of methanolic extract of *Sambucus nigra* (Caprifoliaceae) fruits and leaves

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Received 15-V-2014. Corrected 20-IX-2014. Accepted 22-X-2014.

Abstract: *Toxoplasma gondii* is an obligatory intracellular parasite that infects a wide range of warm-blooded animals and humans. Considering the severity of toxoplasmosis, side effects of current treatments, and the contribution of the ethnopharmacological knowledge for the treatment of parasitic infections, the aim of the present study was to investigate the efficacy of methanolic extracts from the fruits and leaves of *Sambucus nigra* against tachyzoite of *T. gondii*. For this, fruits and leaves of *S. nigra* were collected from Mazandaran province, Iran, were dried under the shade, and powdered using a commercial electrical blender. For extractions, methanol was used as solvent. Virulent RH strain of *T. gondii* was maintained in mice and macrophages containing tachyzoites were aspirated from the peritoneal cavity. Four concentrations (5, 10, 25 and 50mg/mL) of *S. nigra* extract were incubated with infected macrophages for 30, 60, 120 and 180 minutes and the viability of the tachyzoites were evaluated by trypan blue staining. Results showed that *S. nigra* fruit extracts at the concentrations of 5 and 10mg/mL killed 100% of *T. gondii* tachyzoites after 60 and 120 minutes, respectively; and concentrations of 25 and 50mg/mL killed 100% of the tachyzoites after 30 minutes. Additionally, extract of *S. nigra* leaves, at the concentrations of 5, 10 and 25mg/mL after 180 minutes, and concentration of 50mg/mL after 60 minutes, resulted with the highest efficacy. Our results showed that *S. nigra* has acceptable efficacy *in vitro* and the parasitocidal effect of fruit extract was significantly better than leaf extract. However, *in vivo* efficacy of this extract needs further investigation. Rev. Biol. Trop. 63 (1): 7-12. Epub 2015 March 01.

Key words: anti-Toxoplasma, *Sambucus nigra*, *Toxoplasma gondii*, methanolic extract, *in vitro*.

Toxoplasma gondii is an obligatory intracellular protozoan parasite, with wide prevalence in humans and a broad spectrum of vertebrate hosts. It is attained by ingestion of oocysts from contaminated food or water with feces of cats or the ingestion of tissue cysts in under cooked meat (Petersen, 2007). Toxoplasmosis in most situations is an asymptomatic disease, but in some people, flu-like symptoms, swollen lymph glands, muscle aches and pains

that may last from a few days to several weeks has been experienced. Toxoplasmosis is an endemic disease which emerged as one of the most common opportunistic infections in immunocompromised patients (Weiss, & Dubey, 2009). Especially in people with HIV infection, toxoplasmosis commonly involves the brain and causes encephalitis. It may also cause death in these patients (Nissapatorn, 2009). During pregnancy, *T. gondii* can be



transmitted through the placenta and may result in congenital toxoplasmosis, and probably, fetal death. Affected infants may suffer a variety of serious clinical manifestations at birth, including nervous system involvement, mental retardation, neonatal growth retardation, ocular defects, blindness in later life and other generalized clinical manifestations (Ambroise-Thomas, & Pelloux, 1993).

The recommended drugs for treatment or prophylaxis of toxoplasmosis are Pyrimethamine and Sulfadiazine; however, the available drugs are not completely effective in killing the parasite (Serranti, Buonsenso, & Valentini, 2011). On the other hand, these drugs have side effects such as neutropenia, severe drop of platelet count, thrombocytopenia, leucopenia, elevation in serum creatinine and serum liver enzymes, haematological abnormalities and hypersensitivity reactions (Bosch-Driessen et al., 2002; Silveira et al., 2002; Schmidt et al., 2006). For many centuries, herbal remedies and plant extracts have been used as treatments for ailments from headaches to parasite infections (Jones, 1996). Only in the past 20–30 years, scientists have seriously begun to determine the efficacy of plant-derived traditional remedies and their mode of action. *Sambucus* spp. has been reported to be an insect repellent, anti-hemorrhoid, anti-protozoa, and anti-bacterial treatment. It is also mentioned for treatment of burns and infected wounds, edema, eczema, common cold, inflammation and rheumatism (Ahmadiani, Fereidoni, Semnianian, Kamalinejad, & Saremi, 1998; Yeşilada, Gürbüz, & Shibata, 1999; Rahimi-Esboei, Ebrahimzadeh, Gholami, & Falah-Omrani, 2013). Considering the side effects of anti-*Toxoplasma* drugs and the severity of toxoplasmosis, particularly in the fetus and the contribution of the ethnopharmacological knowledge for the treatment of parasitic infections, it is necessary to investigate on new anti-*Toxoplasma* compounds with high activity, low toxicity, and that are cheaper and more effective. Therefore, the aim of the present study was to survey on the efficacy of methanolic extracts from the fruits and leaves of *S. nigra* on tachyzoites of *T. gondii*.

MATERIALS AND METHODS

Preparation of *S. nigra* extract: The fully ripped fruits and leaves of *S. nigra* (from seven plants of 6 years old; height: 3-4.5m) were collected in October 2012 from Mazandaran province, Iran. This province is in the Caspian Sea littoral and is located in the North part of the country that has moist climate. The plants were dried under shade, and powdered mechanically using a commercial electrical blender (Pars Khazar BG-310P). To obtain the methanolic extract, 150g of fruit and leaves dried powders were added to 350mL of pure methanol (30-35°C) and mixed gradually for 1h using a magnetic stirrer. The obtained solutions were covered and left at room temperature (22-25°C) for 24h. The solutions were stirred again and filtered using Whatman No.1 filter paper and the resulting extracts were concentrated over a rotary vacuum (Heidolph-Laborota 4000 efficient, Germany) at 35°C, until a crude solid extract was obtained. The remaining semisolid material was then freeze-dried at -50°C for 24h (MPS-55 Freeze-drier, Cperon, Korea). The obtained filtrate (14.5g) was placed into a sterile glass container and stored at 4°C for further use (Rahimi-Esboei et al., 2013).

Preparation of parasite: Tachyzoites of the virulent RH strain of *T. gondii* were maintained in Swiss Webster mice by intraperitoneal passage every 3-4 days. The project underwent ethical review and was given approval by the Ethics Committee of Mazandaran University of Medical Sciences. Tachyzoites were counted using a hemocytometer (Asai, & Yasuhiro, 1990). For each experiment, 4×10^6 fresh tachyzoites were added in each tube. Tachyzoites were suspended in sterile Phosphate-Buffered Saline (PBS; pH=7.4) containing 100IU/mL penicillin and 100µg/mL streptomycin (Daryani, Hosseini, & Dalimi, 2003; Dadimoghaddam, Daryani, Sharif, Ahmadpour, & Hossienikhah, 2014).

Effect of *S. nigra* extracts on tachyzoites: In this study, four concentrations (5, 10, 25 and

50mg/mL) of the methanolic extracts from the fruits and leaves of *S. nigra* were assessed for 30, 60, 120 and 180minutes. Two mL of each concentration and 4×10^6 fresh tachyzoites were placed in test tube. The contents of the tubes were gently mixed, then incubated at 37°C for 30, 60, 120 and 180minutes. In the end of each incubation time, two mL of 0.5% trypan blue stain was then added to the settled tachyzoites. Then was smeared on a glass slide, and examined under a light microscope. The percentage of dead tachyzoites was determined by counting 200 tachyzoites. Tubes containing PBS and pyrimethamine in concentration of 100mg/mL were considered as negative and positive control groups, respectively. The experiments were performed in triplicate.

Statistical analysis was performed using SPSS-ver20 software. Differences between test and control groups were analyzed by repeated measures ANOVA test. A *p* value less than 0.05 were considered statistically significant.

RESULTS

The anti-*Toxoplasma* effects of the extract of *S. nigra*'s fruits are summarized in Table 1. This extract at the concentration of 5mg/mL killed 62.71, 90.58 and 100% of tachyzoites after 30, 60 and 120 minutes, respectively. Concentration of 10mg/mL after 60 minutes, and also concentrations 25 and 50mg/mL after 30 minutes killed 100% of the tachyzoites.

The anti-*Toxoplasma* effect of methanolic extracts from the fruits of *S. nigra* was extremely significant compared to the negative control group in all exposure times ($p < 0.05$), and a significant difference was found between the fruit methanolic extract of *S. nigra* and the positive control after 30 and 60minutes in all concentrations. The anti-*Toxoplasma* effects of the extract from *S. nigra*'s leaf were summarized in Table 2. Leaves extract at the concentrations of 5, 10 and 25mg/mL killed more than 90% of the tachyzoites in 180minutes,

TABLE 1
The effect of fruits of *S. nigra* on mortality of *T. gondii* tachyzoites *in vitro*

Groups	Concentration	30 min	60 min	120 min	180 min	P- value
Case	5mg/mL	62.71±19.66	90.58±3.46	100	100	<0.05
	10mg/mL	67.12±1.54	100	100	100	<0.05
	25mg/mL	100	100	100	100	<0.05
	50mg/mL	100	100	100	100	<0.05
Control	Pos. 100mg/mL	15.5±1.96	69.37±8.36	95.46±0.48	98.64±1096	<0.001
	Neg. —	3.5±0.14	3.6±0.07	4±0.21	4.8±0.21	>0.05

Pos. = Positive control group receiving 100mg/mL pyrimethamine.
Neg. = Negative control group receiving PBS.

TABLE 2
The effect of leaves of *S. nigra* on mortality of *T. gondii* tachyzoites *in vitro*

Groups	Concentration	30 min	60 min	120 min	180 min	P- value
Case	5mg/mL	57.51±8.88	73.37±0.12	85.36±1.57	91.58±1.75	<0.05
	10mg/mL	66.88±11.23	80.87±1.81	87.79±2.69	94.16±0.33	<0.05
	25mg/mL	67.15±3.71	87.45±0.91	90.12±1.01	96.29±1.50	<0.05
	50mg/mL	80.42±10.10	94.44±3.93	94.20±0.16	94.91±2.96	<0.05
Control	Pos. 100mg/mL	15.5±1.96	69.37±8.36	95.46±0.48	98.64±1096	<0.001
	Neg. —	3.5±0.14	3.6±0.07	4±0.21	4.8±0.21	>0.05

Pos. = Positive control group receiving 100mg/mL pyrimethamine.
Neg. = Negative control group receiving PBS.



while at the concentration of 50mg/mL, 94% of tachyzoites were killed in 60minutes.

The anti-*Toxoplasma* effect of the methanolic extract from the leaf of *S. nigra* was extremely significant compared to the negative control group in all exposure times ($p < 0.05$), but they did not show any significant difference compared with positive control.

DISCUSSION

Pyrimethamine, sulfadiazine, and leucovorin are recommended by World Health Organization (WHO, 1995) and the Centers for Disease Control and Prevention (CDC) as the standard care for toxoplasmosis (Petersen, 2007; Rodriguez & Szajman 2012). These drugs act through inhibiting folic acid synthesis in *T. gondii*, but as in other chemical drugs these have unpleasant side-effects. Therapeutic plants are the traditional treatment of diseases and they are suggested by WHO (World Health, 1995). The use of therapeutic plants by people in developing countries is common because these products are safe and available at low cost.

Our results showed that *S. nigra* has acceptable efficacy on tachyzoites of *T. gondii* *in vitro* and the tachyzoitocidal effects of fruit were significantly better than leaf. According to our results, there was a significant difference between *S. nigra* extracts and control groups in all exposure times.

Sambucus spp. is a member of the Caprifoliaceae family that contains several ingredients responsible for pharmacological activity. Four species of the genus *Sambucus* are growing in Iran. Of these species, *S. ebulus* and *S. nigra* are extensively seen in the Northern regions.

S. nigra was used primarily as antiviral agent for colds, influenza, and herpes virus infections. Many researchers have also demonstrated that *S. nigra* has immune-modulating, antioxidant, and also in folk medicine, *S. nigra* flower was traditionally recommended as a medication for diabetes (Vlachojannis,

Cameron, & Chrubasik, 2010). Among the chemicals contained in this species the quercetin, anthocyanins, cyanidin-3-sambubioside, hemagglutinin protein *S. nigra* agglutinin III (SNA-III), cyanogenic glycosides including sambunigrin, viburnic acid, vitamins A and C have been reported (Johansen, Andersen, Nerdal, & Aksnes, 1991; Mach et al., 1991; Seigler, 1991).

Gholami, Rahimi-Esboei, Ebrahimzadeh, & Pourhajibagher (2013) showed that *S. ebulus* has a nearly high scolicidal activity at the concentrations of 1, 10, 50 and 100mg/mL after 5, 10, 30 and 60 minutes of application, respectively. In another study, Rahimi Esboei et al. (2013) indicated high anti-giardial activity of methanolic extract of *S. ebulus* fruits *in vitro*.

Artemisia is the most common anti-*Toxoplasma* extract in the world. De Oliveria et al., (2009) indicated that treatment of *T. gondii* tachyzoites with *A. annua* infusion before infection in HFF (Human Foreskin Fibroblast) cells exhibited a dose-response inhibitory curve that reached up to 75% of inhibition. Yang et al., (2011) used aqueous extracts of *Astragalus membranaceus* and *Scutellaria baicalensis* on tachyzoites of *T. gondii* *in vitro*; they concluded that parasites treated with either *A. membranaceus* or *S. baicalensis* had significantly decreased intracellular replication at 72, 96 and 120 hours after treatment.

Gozalbes et al., (2000) showed that among the 24 quinolones tested, 20 had positive activity against *T. gondii* with the IC₅₀s ranged from 0.4mg/mL for trovafloxacin to 100mg/mL for cinoxacin and levofloxacin.

The results of our study showed that methanolic extract of *S. nigra* fruits is an effective anti-*Toxoplasma* agent and it seems to be used as a candidate extract for treatment of toxoplasmosis in future researches. However, the *in vivo* efficacy of this extract needs to be explored. Even though *S. nigra* is edible, it is possible side effects when used as an anti-*Toxoplasma* agent needs more investigation. This is the first report on the anti-*Toxoplasma* activity of *S. nigra*.

ACKNOWLEDGMENTS

This study received financial support (grant no. 91-68) from Deputy of Research, Mazandaran University of Medical Sciences, Sari, Iran.

RESUMEN

Actividad anti-*Toxoplasma* de extractos metanólicos de frutos y hojas de *Sambucus nigra* (Caprifoliaceae). *Toxoplasma gondii* es un parásito intracelular obligatorio que infecta a una gran variedad de animales y seres humanos. Teniendo en cuenta la gravedad de la toxoplasmosis, los efectos secundarios de los tratamientos actuales, y la contribución de los conocimientos etnofarmacológicos para el tratamiento de infecciones parasitarias, el objetivo del presente estudio fue investigar la eficacia de los extractos metanólicos de los frutos y hojas de *Sambucus nigra* contra el taquizoito de *T. gondii*. Para esto, se recogieron frutos y hojas de *S. nigra* en la provincia de Mazandaran, Irán, se secaron a la sombra, y se pulverizaron con una batidora eléctrica comercial. Para las extracciones, se empleó metanol como disolvente. La cepa virulenta RH de *T. gondii* se mantuvo en ratones y los macrófagos con taquizoitos se aspiraron de la cavidad peritoneal. Cuatro concentraciones (5, 10, 25 y 50mg/ mL) de extracto de *S. nigra* se encubaron con los macrófagos infectados durante 30, 60, 120 y 180 minutos y la viabilidad de los taquizoitos se evaluó mediante tinción con azul de tripano. Los resultados mostraron que los extractos de frutos de *S. nigra* en las concentraciones de 5 y 10mg/mL mataron al 100% de los taquizoitos de *T. gondii* después de 60 y 120 minutos, respectivamente; y las concentraciones de 25 y 50mg/mL mataron al 100% de los taquizoitos después de 30 minutos. Además, el extracto de hojas de *S. nigra*, en concentraciones de 5, 10 y 25mg/mL después de 180 minutos, y una concentración de 50mg/mL después de 60 minutos, resultaron más eficientes. Nuestros resultados mostraron que *S. nigra* tiene una eficacia aceptable *in vitro* y el efecto parasiticida del extracto de frutos fue significativamente mejor que el del extracto de hoja. Sin embargo, la eficacia *in vivo* de este extracto necesita más investigación.

Palabras clave: anti-*Toxoplasma*, *Sambucus nigra*, *Toxoplasma gondii*, extracto metanólico, *in vitro*.

REFERENCES

Ahmadiani, A., Fereidoni, M., Semnianian, S., Kamalinedjad, M., & Saremi, S. (1998). Antinociceptive and anti-inflammatory effects of *Sambucus ebulus* rhizome extract in rats. *Journal of Ethnopharmacology*, *61*, 229-235.

- Ambroise-Thomas, P., & Pelloux, H. (1993). Toxoplasmosis - congenital and in immunocompromised patients: A parallel. *Parasitology Today*, *9*, 61-63.
- Asai, T., & Yasuhiro, S. (1990). Remarkable activities of nucleoside triphosphate hydrolase in the tachyzoites of both virulent and avirulent strains of *Toxoplasma gondii*. *FEMS microbiology letters*, *72*, 89-92.
- Bosch-Driessen, L. H., Verbraak, F. D., Suttorp-Schulten, M. S. A., van Ruyven, R. L. J., Klok, A. M., Hoyng, C. B., & Rothova, A. (2002). A prospective, randomized trial of pyrimethamine and azithromycin vs pyrimethamine and sulfadiazine for the treatment of ocular toxoplasmosis. *American Journal of Ophthalmology*, *134*, 34-40.
- Dadimoghaddam, Y., Daryani, A., Sharif, M., Ahmadpour, E., & Hossienikhah, Z. (2014). Tissue tropism and parasite burden of *Toxoplasma gondii* RH strain in experimentally infected mice. *Asian Pacific Journal of Tropical Medicine*, *7*, 521-524.
- Daryani, A., Hosseini, A. Z., & Dalimi, A. (2003). Immune responses against excreted/secreted antigens of *Toxoplasma gondii* tachyzoites in the murine model. *Veterinary Parasitology*, *113*, 123-134.
- De Oliveira, T. C., Silva, D. A. O., Rostkowska, C., Bla, S. R., Ferro, E. A. V., Magalhes, P. M., & Mineo, J. (2009). *Toxoplasma gondii*: Effects of *Artemisia annua* L. on susceptibility to infection in experimental models *in vitro* and *in vivo*. *Experimental Parasitology*, *122*, 233-241.
- Gholami, S. H., Rahimi-Esboei, B., Ebrahimzadeh, M. A., & Pourhajibagher, M. (2013). *In vitro* effect of *Sambucus ebulus* on scolices of Hydatid cysts. *European Review for Medical and Pharmacological Sciences*, *17*, 1760-1765.
- Gozalbes, R., Brun-Pascaud, M., Garcia-Domenech, R., Galvez, J., Girard, P. M., Doucet, J. P., & Derouin, F. (2000). Anti-*Toxoplasma* activities of 24 quinolones and fluoroquinolones *in vitro*: prediction of activity by molecular topology and virtual computational techniques. *Antimicrobial Agents and Chemotherapy*, *44*, 2771-2776.
- Johansen, O. P., Andersen, O. M., Nerdal, W., & Aksnes, D. W. (1991). Cyanidin 3-[6-(p-coumaroyl)-2-(xylosyl)-glucoside]-5-glucoside and other anthocyanins from fruits of *Sambucus canadensis*. *Phytochemistry*, *30*, 4137-4141.
- Jones, F. A. (1996). Herbs-useful plants. Their role in history and today. *European Journal of Gastroenterology & Hepatology*, *8*, 1227-1231.
- Mach, L., Scherf, W., Ammann, M., Poetsch, J., Bertsch, W., Marz, L., & Glossl, J. (1991). Purification and partial characterization of a novel lectin from elder (*Sambucus nigra* L.) fruit. *Biochemical Journal*, *278*, 667-671.



- Nissapatorn, V. (2009). Toxoplasmosis in HIV/AIDS: a living legacy. *The Southeast Asian Journal of Tropical Medicine and Public Health*, 40, 1158-1178.
- Petersen, E. (2007). Toxoplasmosis. *Seminars in Fetal and Neonatal Medicine*, 12, 214-223.
- Rahimi-Esboei, B., Ebrahimzadeh, M. A., Gholami, S., & Falah-Omrani, V. (2013). Anti-giardial activity of *Sambucus ebulus*. *European Review for Medical and Pharmacological Sciences*, 17, 2047-2050.
- Rodriguez, J. B., & Szajnman, S. H. N. (2012). New anti-bacterials for the treatment of toxoplasmosis; a patent review. *Expert Opinion on Therapeutic Patents*, 22, 311-333.
- Schmidt, D. R., Hogh, B., Andersen, O., Hansen, S. H., Dalhoff, K., & Petersen, E. (2006). Treatment of infants with congenital toxoplasmosis: tolerability and plasma concentrations of sulfadiazine and pyrimethamine. *European Journal of Pediatrics*, 165, 19-25.
- Seigler, D. S. (1991). Cyanide and cyanogenic glycosides. *Herbivores: Their interactions with secondary plant metabolites*, 1, 35-77.
- Serranti, D., Buonsenso, D., & Valentini, P. (2011). Congenital toxoplasmosis treatment. *European Review for Medical and Pharmacological Sciences*, 15, 193-198.
- Silveira, C., Belfort Jr, R., Muccioli, C., Holland, G. N., Victora, C. G., Horta, B. L., & Nussenblatt, R. B. (2002). The effect of long-term intermittent trimethoprim/sulfamethoxazole treatment on recurrences of toxoplasmic retinochoroiditis. *American Journal of Ophthalmology*, 134, 41-46.
- Vlachojannis, J. E., Cameron, M., & Chrubasik, S. (2010). A systematic review on the sambuci fructus effect and efficacy profiles. *Phytotherapy Research*, 24, 1-8.
- Weiss, L. M., & Dubey, J. P. (2009). Toxoplasmosis: A history of clinical observations. *International Journal for Parasitology*, 39, 895-901.
- World Health Organization. (1995). WHO model prescribing information: drugs used in parasitic diseases, *World Health Organization*.
- Yang, X., Huang, B., Chen, J., Huang, S., Zheng, H., Lun, Z. R., & Lu, F. (2011). In vitro effects of aqueous extracts of *Astragalus membranaceus* and *Scutellaria baicalensis* GEORGI on *Toxoplasma gondii*. *Parasitology Research*, 110, 2221-2227.
- Yeşilada, E., Gürbüz, I., & Shibata, H. (1999). Screening of Turkish anti-ulcerogenic folk remedies for anti-*Helicobacter pylori* activity. *Journal of Ethnopharmacology*, 66, 289-293.

