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SELECTIVE *IN VITRO* GROWTH INHIBITORY EFFECT OF *WITHANIA SOMNIFERA* ON HUMAN PATHOGENIC BACTERIA AND BIFIDOBACTERIA

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Abstract

Antimicrobial activity of crude acetone extract from the aerial part of *Withania somnifera* was tested *in vitro* against six pathogenic bacteria viz., *Bacillus cereus*, *B. subtilis*, *Staphylococcus epidermis*, *S. aureus*, *Streptococcus pneumoniae* and *S. pyogenes* and five bifidobacteria viz., *Bifidobacterium animalis*, *B. breve*, *B. catenulatum*, *B. infantis* and *B. longum* evaluating its potential harmful effect on beneficial gastrointestinal microbiota using disk diffusion method. The results showed that pathogenic bacteria were significantly more susceptible to the extract of *W. somnifera* than bifidobacteria (average of diameters of inhibition zones 20.45 mm and 13.10 mm, respectively) at the concentration of 2 mg/disc. Among all bacteria tested, *S. pneumoniae* was the most sensitive, while *B. animalis* was the most resistant species. These results suggest *W. somnifera* as an effective antibacterial agent against human pathogenic bacteria with lowered harmful effect on bifidobacteria.

Introduction

Due to the increasing drug resistance, emergence of uncommon infections and appearance of undesirable effects of antibiotics during the last years, the antimicrobial properties of plant-derived products have been reported more frequently with attempt to discover new chemical classes of safer antimicrobial agents (Rabanal *et al*., 2002, Walter *et al*., 2011). In this context, higher plants are nowadays known to effectively inhibit wide range of microbial pathogens, especially Gram-positive bacteria (Rios & Recio, 2005, Shinwari *et al*., 2009). Nevertheless, some of the aspects of antibacterial action of plant extracts and compounds, e.g. harmful adverse effect on beneficial human microbiota, are still poorly known.

Withania somnifera (L.) Dunal (Solanaceae), also known as Ashwagandha, Indian ginseng or Winter Cherry (Andallu & Radhika, 2000, Yousaf *et al*., 2008) is an evergreen shrub, widely grown in India as one of the most important herbs in Ayurvedic medicine. Roots and leaves of this species have traditionally been used, among its many other applications, as an anti-infective remedy against a number of health disorders such as ulcers, rashes, painful swellings, septic wounds, gonorrhoea, syphilis, conjunctivitis, stomach-ache, respiratory problems, colds or tuberculosis (Kurup, 1958; Uma Devi *et al*., 1996; Gupta & Rana, 2007; Yousaf *et al*., 2008). The modern pharmacological studies subsequently confirmed marked antimicrobial action of the plant in several previously published papers (Kurup, 1956; Bhatnagar *et al*., 1961; Budhiraja & Sudhir, 1987; Owais *et al*., 2005). The various kinds of extracts e.g., aqueous, ethanol, methanol, petrol, and chloroform of the leaves and roots possessed *in vitro* growth-inhibitory activity effect against various pathogenic microorganisms, whereas leaf extracts showed much stronger activity than the roots (Kurup, 1958; Jaffer *et al*., 1988) being reported to effectively inhibit the growth of *Bacillus subtilis, Diplococcus pneumoniae, Escherichia coli, Micrococcus pyogenes, Salmonella typhi*, *S. typhimurium*, *Shigella dysenteriae, Staphylococcus aureus,* and *Streptococcus pyogenes* (Kurup, 1958, 1956; Bhatnagar *et al*., 1961; Ikram & Haq, 1980; Jaffer *et al*., 1988;Owais *et al*., 2005). Moreover, the i*n vivo* studies suggest that leaf aqueous extracts was efficient in the treatment of experimental systematic salmonellosis in mice (Owais *et al*., 2005). Withanolides, a class of constituents belonging to the group of steroidal lactones, have been reported as main antimicrobial compounds of the leaf extract (Sethi *et al*., 1974; Atta-ur-Rahman *et al*., 1993; Singh & Kumar, 1998; Mishra *et al*., 2000; Ganzera *et al*., 2003; Jayaprakasam *et al*., 2003) with significant inhibitory effect against various pathogenic bacteria such as *S. aureus, B. subtilis, E. coli, Pseudomonas aeruginosa,* and *S. typhimurium* (Kurup, 1956; Sethi *et al*., 1974; Owais *et al*., 2005).

Although the number of studies evaluating antibacterial activity of withanolides (Kurup, 1958; Sethi *et al*., 1974; Budhiraja & Sudhir, 1987; Shanazbanu Shashidara & Babu, 2006) and *W. somnifera* leaf extracts (Kurup, 1956; Jaffer *et al*., 1988; Singh & Kumar, 1998; Owais *et al*., 2005) have been conducted in the past, according to our best knowledge, no efforts have been made to investigate effect of *W. somnifera* extracts on growth of positive intestinal microbiota. Thus, we decided to take up detailed investigation of *W. somnifera* acetone extract, which showed promising antimicrobial action in our previous experiments (Polesny *et al*., 2008), and evaluate its selective *in vitro* inhibitory effect on the growth of several strains of Gram-positive bacteria and bifidobacteria.

Materials and Methods

Plant material: The seeds of *W. somnifera* were obtained from the Svinviks Arboretum, Ringve Botanical Garden, Norwegian University of Science and Technology (Trondheim, Norway) and were grown in the experimental field of the Institute of Tropics and Subtropics of the Czech University of Life Sciences Prague (ITS CULS Prague). Leaves of the plant were collected during the months August-October 2001 and authenticated by Dr. Zbynek Polesny from ITS CULS Prague. Voucher specimen has been deposited at ITS CULS Prague under the collection number POL 373.

Preparation of extract: Dried plant material (15.0 g) was finely ground and macerated at room temperature in 450 ml of acetone p.a. (Lach-Ner, CZ) for 5 days. The extract was afterwards filtered and concentrated *in vacuo* at 40°C. The dried residue (yield 5.73% w/w) was resolved in dimethyl sulfoxide (DMSO) (Lach-Ner, CZ) and then diluted in Tris buffered saline (TBS) of pH 7.6 (Sigma-Aldrich, CZ) to create the stock solution of the extract in concentration of 100 mg/ml in 1% DMSO. Finally, the extract was sterilized by filtration through a 0.2 µm membrane filter (Sartorius, DE) and stored at +4°C until tested.

Microorganisms tested: The following American Type Culture Collection (ATCC) bacterial strains were used: *Bacillus cereus* ATCC 11778, *B. subtilis* ATCC 6633, *Bifidobacterium animalis* ATCC 25527, *B. catenulatum* ATCC 27539, *B. breve* ATCC 15700, *B. infantis* ATCC 17930, *B. longum* ATCC 15707, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATTC 27853, *Staphylococcus aureus* ATCC 25923, *S. epidermis* ATCC 12228, *Streptococcus pneumoniae* ATCC 6305, and *S. pyogenes* ATCC 19615. The pathogenic bacteria were grown on Mueller-Hinton agar, supplemented with 5% of defibrinated horse blood for testing of streptococci. Bifidobacteria were grown on Wilkins-Chalgren anaerobe agar under anaerobic conditions using Anaerobic Jar HP11 (Oxoid, UK). All cultivation media, microbial strains and antibiotic were purchased from Oxoid, UK.

Antimicrobial assay: The antibacterial activity of the crude plant extract was determined using a disk diffusion method (Jorgensen *et al*., 1999). Each Petri dish containing appropriate nutrient agar was inoculated with 100 µl of broth adjusted to 10^7 CFU/ml of bacterial cell suspension and then the inoculum was spread across the surface of the plate using a sterile glass spreader. The sterile filter paper disc (Oxoid, UK) of 6 mm in diameter was loaded with 2 mg of extract using pipette, dried under laminar air flow conditions and positioned on previously inoculated plates. After 24 hours of incubation under the stated conditions at 37°C, the diameters of the growth inhibition zones were measured and recorded in mm at all plates. The susceptibility of all bacterial strains to reference antibiotic (5 µg/disc of vancomycin) was evaluated, simultaneously. The disks saturated with 1% of DMSO assayed as the negative control, did not affect growth of any bacteria tested.

Statistical analysis: Each value represents mean ± standard deviation obtained from 3 replicates. Statistical significance of differences was calculated using Student's *t*-test and $p<0.05$ was considered as significant.

Results and Discussion

W. somnifera leaf acetone extract possessed a selective effect within Gram-positive bacteria (Table 1), whereas growth of potential pathogens (average of inhibition zones 20.45 ± 1.34 mm) was significantly ($p<0.05$) more affected than the growth of bifidobacteria (average of inhibition zones 13.10 ± 1.22 mm). Considering the pathogenic species, the most sensitive and resistant bacteria were *S. pneumoniae* and *S. aureus* with inhibition zones of 25.00 ± 3.61 mm and 14.33 ± 0.58 mm, respectively. Among other bacterial strains, *S. pyogenes* and *S. epidermis* were also markedly inhibited with inhibition zones exceeding 21 mm in diameter. With exception of *B. infantis*, all bifidobacterial strains exhibited relatively high level of resistance to the *W. somnifera* extract, whereas *B. animalis* showed the most pronounced resistance with the growth-inhibition zone diameter of 9.50 ± 0.71 mm. Other bifidobacteria, viz., *B. breve*, *B. catenulatum*, and *B. longum*, were only slightly more affected with diameters of inhibition zones ranging from 13.5 to 14.00 mm. In the positive control test, all bacterial strains were susceptible to vancomycin at a concentration of 5 µg per disk. The pathogenic bacteria tend to be more tolerant to reference antibiotic with average of inhibition zones 17.45 ± 0.79 mm, whereas the effect on bifidobacterial growth was even significantly higher ($p<0.05$) with average of inhibition zones 21.20 ± 4.12 mm.

Table 1. Antibacterial activity of *W. somnifera* acetone extract on pathogenic bacteria and bifidobacteria.

Microorganisms	Inhibition zone (mm)[a]	
	Extract[b]	Vancomycin[c]
Bacillus cereus	14.33 ± 0.58	12.00 ± 1.00
Bacillus subtilis	19.67 ± 0.58	12.33 ± 0.58
Staphylococcus aureus	21.33 ± 0.58	14.67 ± 0.58
Staphylococcus epidermis	25.00 ± 3.61	23.00 ± 2.00
Streptococcus pneumoniae	21.67 ± 1.15	23.00 ± 0.00
Streptococcus pyogenes	20.67 ± 1.53	19.67 ± 0.58
Average	20.45 ± 1.34	17.45 ± 0.79
Bifidobacterium animalis	9.50 ± 0.71	21.00 ± 4.24
Bifidobacterium breve	14.00 ± 1.15	21.00 ± 5.03
Bifidobacterium catenulatum	13.50 ± 2.12	23.00 ± 4.24
Bifidobacterium infantis	17.00 ± 0.00	21.00 ± 2.83
Bifidobacterium longum	13.50 ± 2.12	20.00 ± 4.24
Average	13.10 ± 1.22	21.20 ± 4.12

[a]Mean ± standard deviation obtained from 3 replicates. [b]*W. somnifera* acetone extract (2 mg/disk). [c]Antibiotic positive reference standard (5 µg/disk)

Intestinal tract in a healthy man is a complex ecosystem of various microorganisms, predominantly composed of bifidobacteria and lactic acid bacteria, which play an important role not only in metabolism, but also in host defence against infection, aging, and immuno-potentiation (Ahn *et al*., 1990a; 1990b; 1994). Intake of conventional antibiotics is known to cause disturbance of intestinal microbiota, which leads to various diseases or abnormal physiological states (Ahn *et al*., 1991; Lee & Kim, 2002). There are literally thousands of published scientific papers from around the globe describing the antimicrobial activities of plant extracts and their chemical constituents (Rios & Recio, 2005), but only few of them deal with selective antibacterial action to intestinal microbiota.

Our findings demonstrate selective antibacterial activity of *W. somnifera* leaf acetone extract, which correspond with results of previously published papers reporting similar effects of various plant-derived products (extracts or compounds) against intestinal and pathogenic bacteria (Ahn *et al*., 1990a; 1990b; 1991; 1994; 1998; Puupponen-Pimia *et al*., 2001; 2005; Lee & Kim, 2002; Kamijo *et al*., 2008). For instance, Ahn *et al*., (1990a; 1990b) studied ginseng (*Panax ginseng*), whose aqueous and methanol extracts showed to promote the growth of 27 bifidobacteria strains such as *B. adolescentis, B. longun, B. breve,* and *B. infantis* and selectively inhibit the growth of several clostridia strains and *E. coli*. Interestingly, ginsenosides, the active constituents of *P. ginseng* were reported to be similar to withanolides in their structure and activity (Grandhi *et al*., 1994), which suggest possible involvement of withanolides present in *W. somnifera* leaf extracts also for its selective antibacterial action. In another study (Ahn *et al*., 1991), polyphenols from green tea (*Thea chinensis*) were reported as selective inhibitors of several clostridia strains, while enhancing the growth of bifidobacteria. Kamijo *et al*., (2008) described that pulverized petals of *Rosa rugosa* showed selective antibacterial activity inhibiting the growth of *Bacteroides bulgatus, E. coli, S. aureus, B. cereus,* and *Salmonela* sp.*,* whereas only slightly affected the growth of *B. breve* and *Lactobacillus salivarius*. Similarly, Puupponen-Pimia *et al*., (2001; 2005) studied several extracts of Nordic berries such as cloudberry (*Rubus chamaemorus*) or raspberry (*R. idaeus*), which have been observed to possess inhibitory effect against both Gram-positive and Gram-negative pathogenic bacteria without affecting the growth of *Lactobacillus* species. As a result, phenolic compounds were suggested as main substances responsible for the antibacterial action in the studies mentioned above (Ahn *et al*., 1991; Puupponen-Pimia *et al*., 2001; 2005; Kamijo *et al*., 2008). Lee & Kim (2002) have previously isolated flavonoids; quercetin and kaempferol glycosides from *Ginkgo biloba* leaves, which possessed strong antibacterial activity against numerous bacterial strains, while bifidobacteria and lactobacilli were not inhibited. In general, flavonoids have been widely studied for their effective antibacterial activity (Cushnie & Andrew, 2005). Since various flavonoids, including quercetin glycosides were also found in the leaves of *W. somnifera* (Kandil *et al*., 1994) we suppose that flavonoid or other phenolic compounds may participate in selective inhibitory action of the extract tested.

In conclusion, we can summarize that the acetone extract of *W. somnifera* leaves possessed significant selective antibacterial action on potential human pathogenic bacteria and intestinal bifidobacteria. Based on previously described presence of withanolides together with flavonoids in the leaves of *W. somnifera* we suggest that these compounds might be responsible for selective antibacterial activity of the plant. However, further phytochemically and pharmacologically oriented research is necessary to identify the specific active constituents of *W. somnifera* leaf acetone extract. Considering the previously reported low toxicity (Kulkarni & Ashish Dhir, 2008) and effective growth-inhibiting effects against pathogenic bacteria and bifidobacteria observed in our study, we suggest *W. somnifera* as the prospective antibacterial agent with future potential application in food or pharmaceutical industry.

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References

Ahn, Y.J., J.H. Kwon, S.H Chae, J.H. Park and J.Y. Yoo. 1994. Growth-inhibitory responses of human intestinal bacteria to extracts of oriental medicinal plants. *Microb. Ecol. Health D.,* 7: 257-261.

Ahn, Y.J., M.J. Kim, T. Kawamura, T. Yamamoto, T. Fujisawa and T. Mitsuoka. 1990a. Effect of *Panax ginseng* extract on growth responses of human intestinal bacteria and bacterial metabolism. *Korean J. Ginseng Sci*., 14: 253-264.

Ahn, Y.J., M.J. Kim, T. Yamamoto, T. Fujisawa and T. Mitsuoka. 1990b. Selective growth responses of human intestinal bacteria to Araliaceae extracts. *Microb. Ecol. Health D.,* 3: 223-229.

Ahn, Y.J., S.J. Park, D.H. Choi, H.Ch. Cho and I.G. Hiremath. 1998. Growth-inhibitory responses of human intestinal bacteria to extracts from Indian and African plants. *Agr. Chem. Biotechnol.,* 41(1): 104-109.

Ahn, Y.J., T. Kawamura, M. Kim, T. Yamamoto and T. Mitsuoka. 1991. Tea polyphenols: selective growth inhibitors of *Clostridium* spp. *Agric. Biol. Chem.*, 55(5): 1425-1426.

Andallu, B. and B. Radhika. 2000. Hypoglycemic diuretic and hypocholesterolemic effect of white cherry (*Withania somnifera* Dunal) root. *Indian J. Exp. Biol*., 38(6): 607-609.

Atta-ur-Rahman Abbas, S., A.S. Dur-e-Shahwar Jamal and I.M. Choudhary. 1993. New withanolides from *Withania* sp. *J. Nat. Prod*., 56(7): 1000-1006.

Bhatnagar, S.S., H. Santapau, J.D.H. Desa, A.C. Maniar, N.C. Chadially, M.J. Solomon, S. Yellore and T.N.S. Rao. 1961. Biological activity of Indian medicinal plants Part. I. *Ind. J. Med. Res*., 49: 799-813.

Budhiraja, R.D. and S. Sudhir. 1987. Review of biological activity of withanolides. *J. Sci. Ind. Res.*, 46(11): 488-491.

Cushnie, T.T.P. and J.L. Andrew. 2005. Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Agents*, 26(5): 343-356.

Ganzera, M., M.I. Choudhary and I.A. Khan. 2003. Quantitative HPLC analysis of withanolides in *Withania somnifera*. *Fitoterapia*, 74(1-2): 68-76.

Grandhi, A., A.M. Mujumdar and B. Parwardhan. 1994. A comparative pharmacological investigation of Ashwagandha and Ginseng. *J. Ethnopharmacol*., 44(3): 131-135.

Gupta, L.G. and A.C. Rana. 2007. PHCOG MAG.: Plant review. *Withania somnifera* (Ashwagandgha): *A Review. Pharmacol. Rev.*, 1(1): 129-136.

Ikram, M. and I. Haq. 1980. Screening of medicinal plants for antimicrobial activity. Part I. *Fitoterapia*, 51: 231-235.

Jaffer, H.J., A.L.M. Jawad, H.S. Saber and A. Al-Naib. 1988. Evaluation of antimicrobial activity of *Withania somnifera* extracts. *Fitoterapia*, 59(6): 497-500.

Jayaprakasam, B., Y. Zhang, P.N. Seeram and M.G. Nair. 2003. Growth inhibition of human tumour cell lines by withanolides from *Withania somnifera* leaves. *Life Sci.*, 74(1): 125-132.

Jorgensen, J.H., J.D. Turnidge and J.A. Washington. 1999. Antibacterial susceptibility tests: Dilution and disk diffusion methods. In: *Manual of clinical microbiology*, 7th ed. (Eds.): P.R. Murray, E.J. Baron, M.A. Pfaller, F.C. Tenover and R.H. Yolken. ASM Press, 1526-1543.

Kamijo, M., T. Kanazawa, M. Funaki, M. Nishizawa and T. Yamagishi. 2008. Effects of *Rosa rugosa* petals on intestinal bacteria. *Biosci. Biotechnol Biochem*., 72(3): 773-777.

Kandil, F.E., N.H. El-Sayed, A.M. Abou-Douh, M.S. Ishak and T.J. Mabry. 1994. Flavonol glycosides and phenolics from *Withania somnifera. Phytochemistry*, 37(4): 1215-1216.

Kulkarni, S.K. and Ashish Dhir. 2008. *Withania somnifera*: An Indian ginseng. *Prog. Neuropsychopharmacol. Biol. Psychiatry*, 32(5): 1093-1105.

Kurup, P.A. 1956. The antibacterial principle of the leaves of *Withania somnifera. Curr. Sci.,* (Bangalore) 25: 57-58.

Kurup, P.A. 1958. The antibacterial principle of *Withania somnifera.* I. Isolation and antibacterial activity. *Antibiot. Chemother.*, 8: 511-515.

Lee, H.S. and M.J. Kim. 2002. Selective responses of three *Ginkgo biloba* leaf-derived constituents on human intestinal bacteria. *J. Agric. Food Chem*., 50(7): 1840-1844.

Mishra, L.C., B.B. Singh and S. Dagenais. 2000. Scientific basis for the therapeutic use of *Withania somnifera* (Ashwagandha): a review. *Altern. Med. Review*, 5: 334- 346.

Owais, M., K.S. Sharad, A. Shehbaz and M. Saleemuddin. 2005. Antibacterial efficacy of *Withania somnifera* (Ashwagandha) and indigenous medicinal plant against experimental murine salmonellosis. *Phytomedicine*, 12(3): 229-235.

Polesny, Z., J. Flesar, I. Jankovska, V. Rada and L. Kokoska. 2008. Selective *in vitro* inhibitory effect of *Withania somnifera* extract on growth of human pathogenic bacteria and bifidobacteria. First European Food Congress ´08, Ljubljana, Slovenia, Abstract p. 030.

Puupponen-Pimia, R., L. Nohynek, C. Meier, M. Kahkonen, M. Heinone, A. Hopia and K.M. Oksman-Caldentey. 2001. Antimicrobial properties of phenolic compounds from berries. *J. Appl. Microbiol*., 90(4): 494-507.

Puupponen-Pimia, R., L. Nohynek, S. Hartmann-Schmidlin, M. Kahkonen, M. Heinonen, K. Maatta-Riihinen and K.M. Oksman-Caldentey. 2005. Berry phenolics selectively inhibit the growth of intestinal pathogens. *J. Appl. Microbiol.*, 98(4): 991-1000.

Rabanal, R.M., A. Arias, B. Prado, M. Hernández-Pérez and C.C. Sánchez-Mateo. 2002. Antimicrobial studies on three species of *Hypericum* from the Canary Islands. *J Ethnopharmacol*., 81(2): 287-292.

Rios, J.L. and M.C. Recio. 2005. Medicinal plants and antimicrobial activity. *J Ethnopharmacol*., 100(1-2): 80-84.

Sethi, P.D., K.B. Sharma and S. Subramanian Sankara. 1974. Antibacterial activity of some C28 steroidal lactones. *Indian. J. Pharm*., 36: 122-123.

Shanazbanu Shashidara, S. and V.L.A. Babu. 2006. Isolation of withaferin-A from *Withania somnifera* Dun. leaves and its antibacterial activity. *Asian J. Chem*., 18(2): 1243-1247.

Shinwari, Z.K., I. Khan, S. Naz and A. Hussain. 2009. Assessment of antibacterial activity of three plants used in Pakistan to cure respiratory diseases. *Afr. J. Biotechnol.*, 8(24): 7082-7086

Singh, S. and S. Kumar. 1998. "*Withania somnifera*: the Indian ginseng Ashwagandha", 1st Ed. Central institute of medicinal and aromatic plants (CIMAP), Lucknow, India, 131-201 pp.

Uma Devi, P., K. Akagi, V. Ostapenko, Y. Tanaka and T. Sugahara. 1996. Withaferin A: a new radiosensitizer from the Indian medicinal plant *Withania somnifera*. *Int. J. Radiat. Biol.*, 69(2): 193-197.

Walter, C., Z.K. Shinwari, I. Afzal and R.N. Malik. 2011. Antibacterial activity in herbal products used in Pakistan. *Pak. J. Bot*., 43 (Special Issue): 155-162.

Yousaf, Z., S. Masood, Z.K. Shinwari, M.A. Khan and A. Rabani. 2008. Evaluation of taxonomic status of medicinal species of the genus *Hyoscyamous,Withania, Atropa* and *Datura* based on poly acrylamide gel electrophoresis. *Pak. J. Bot.*, 40(6): 2289-2297.

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