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# Inhibitory effect of different solvent extracts of *Vitex negundo* L. and *Allium sativum* L. on phytopathogenic bacteria

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## ABSTRACT

*There is a worldwide interest in searching for the safe and effective novel antibacterial compounds of plant origin for the control of plant pathogenic bacteria which is responsible for the great impact on the growth and productivity of agriculture crops. In this study an attempt was made to determine the in vitro antibacterial activity of sequentially extracted different solvent (dichloromethane, ethyl acetate, ethanol, methanol and water) extracts of leaf, flower and fruit of Vitex negundo L. and bulb of Allium sativum L. (Garlic) against phytopathogens namely Pseudomonas solanacearum and Xanthomonas axonopodis pv. citri. The preliminary antibacterial activity was performed by agar well diffusion method and the minimum inhibitory concentration (MIC) values were determined by agar dilution method. The test samples were also subjected to qualitative phytochemical analysis. One way analysis of variance (ANOVA) followed by least significant difference (LSD) test were done for the statistical analysis of the data. All the test samples showed inhibitory effect on both of the test pathogens and the diameter of inhibition zone ranged from 9.9 ± 0.5 mm to 48.5 ± 1.3 mm and the inhibitory effect differed significantly ($P<0.05$) among the samples. Ethyl acetate extract of flower of Vitex negundo L. showed significantly ($P<0.05$) higher inhibition on Pseudomonas solanacearum and Xanthomonas axonopodis pv. citri. The MIC values of ethyl acetate extracts of fruit and flower of Vitex negundo L. and Allium sativum and ethanol extract of flower of Vitex negundo L. ranged from 2.5 mg / ml to 40 mg / ml. Phytochemical analysis of above extracts revealed the presence of alkaloids, flavonoids, tannins, cardiac glycosides and terpenoids. Further studies are being carried out to elucidate the active principles responsible for the inhibitory effect of these pathogens and to determine their activity in vivo. This is the first report that reveals the inhibitory effect of Vitex negundo L. on Pseudomonas solanacearum and Xanthomonas axonopodis pv. citri.*

**Key words:** Antibacterial activity, *Vitex negundo, Allium sativum,* plant pathogens.

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## INTRODUCTION

Synthetic pesticides are nowadays widely used for the control of plant diseases throughout the world because of their higher effectiveness in controlling disease causing organisms. However, excessive and unsystematic application of these chemicals has created several environmental and health hazards and also some phytopathogens have been developed resistance [1]. Therefore, there is an urgent need to search for effective, safe and biodegradable alternative pesticides.

Green plants are found to be an effective reservoir for the bioactive molecules and can provide valuable sources for the discovery of natural pesticides [2]. Therefore in recent years medicinal plant extracts are intensively analyzed with an aim of isolating novel bioactive compounds.

*Vitex negundo* L. (Verbenaceae) is a medicinal plant found in the low country and coastal areas in Sri Lanka [3]. This plant is used for the treatment of dengue, rheumatism, dyspepsia and diarrhea [4]. *Allium sativum* L. (Liliaceae) is a bulbous herb and it is cultivated in upcountry districts in Sri Lanka [3], and it has a long folkloric history as a treatment for cold, cough, asthma and also it strengthen the immune system [5].

Although several studies have been carried out to report the antibacterial effect of these plants on human pathogenic bacteria, a few report about *Allium sativum* and no report about *Vitex negundo* reveled the inhibitory effect on *Xanthomonas axonopodis* pv. c*itri* and *Pseudomonas solanacearum*. Therefore in the present study an attempt has been made to test the effectiveness of *in vitro* growth control of sequentially extracted different solvent extracts of leaf, flower and fruit of *Vitex negundo* and bulb of *Allium sativum* against two phytopathogens *Xanthomonas axonopodis* pv. c*itri* and *Pseudomonas solanacearum* and also qualitative phytochemical screening was performed to predict the active chemicals present in the test samples.

## MATERIALS AND METHODS

### Collection and identification of plant materials

Fresh and disease free leaf, flower and fruit of *Vitex negundo* were collected from Jaffna, North of Sri Lanka and was identified and authenticated with the herbarium at the Department of Botany, University of Jaffna, Sri Lanka. Plant materials were washed thoroughly three times with running tap water and once with sterile distilled water and then dried under shade. Fresh and healthy bulbs of *Allium sativum* (Garlic) were purchased from local medical shop and were peeled, cut into pieces and sun dried. Then the dried materials were ground to fine powder using an electric blender and the powder of both were stored in airtight dark bottles until further used.

### Preparation of extracts

Powdered materials were successively extracted using solvents of increasing polarity [6]. 50g powder was initially soaked in 200 ml of DCM in airtight conical flask in a shaker for 72 hours and then it was first filtered through doubled layered muslin cloth and then filtered through Whatman No 1filter paper. The filtrate was collected into airtight brown bottle, similar process was repeated thrice with fresh DCM and the filtrates were pooled together. Finally DCM was removed from the filtrate under reduced pressure using rotary evaporator at low temperature and the dried extract was stored in the refrigerator. The residue was dried and used for the ethyl acetate extraction followed by ethanol, methanol and water similar to the procedure carried out for the DCM extraction.

### Phytochemical analysis

A portion of the each test samples were subjected to preliminary qualitative phytochemical screening for the presence of alkaloids, flavonoids, cardiac glycosides, terpenoids, tannins and saponins [7].

### Test Bacteria

Bacteria namely *Xanthomonas axonopodis* pv. c*itri* and *Pseudomonas solanacearum* were isolated from infected citrus plant (*Citrus aurantifolia*) and tomato plant (*Solanum lycopersicum*) respectively, and identified based on their physiological and biochemical

characteristics [8,9]. The bacteria were maintained on nutrient agar slant at 4 °C and sub cultured periodically on fresh medium.

**Determination of antibacterial activity**

Antibacterial activity of the test samples was tested by agar well diffusion method according to previous study [10], briefly, the test bacteria were cultured in nutrient broth at 28 °C for 24 hours and the fresh inoculums were taken for the test. Autoclaved Mueller Hinton agar (MHA) medium was cold down to 40 °C, and then 1ml of bacterial suspension ($10^6$ cfu / ml) was mixed with 15 ml of this medium, poured into a sterile Petri dish and allowed to set. Wells were then made using a sterile cork borer (8mm) and filled with 100 µl of each extract (50mg / 100µl) dissolved in the mixture of dimethyl sulfoxide (DMSO) and acetone (1:1 v/v). Streptomycin (50 µg / 100 µl) and 100 µl of mixture of DMSO and acetone were used as standard and control respectively. Plates were incubated at 28 °C and the antibacterial activity was determined by measuring the diameter of zone of inhibition.

**Determination of Minimum inhibitory concentration (MIC)**

Ethyl acetate extract of fruit and flower of *Vitex negundo* and bulb of *Allium sativum* and ethanol extract of flower of *Vitex negundo* were selected to find out the Minimum Inhibitory Concentration (MIC) values by agar dilution method [11]. The extracts were added at concentrations of 0(control), 1.25, 2.5, 5, 10, 20, 40, 80 and 160 mg/ml in molten Mueller Hinton agar and poured in Petri dishes. The overnight culture was spot-inoculated on the plates such that each inoculum contained $10^6$ CFU. Plates were incubated at 28°C and examined after 24 hours.

**Statistical Analysis**

The results of the antibacterial activity of the replicates were expressed as mean ± standard deviation (SD) and the data were subjected to examine by analysis of variance (ANOVA) ($P<0.05$) followed by least significant difference (LSD) test ($\alpha = 0.05$) by using a software, SAS system for windows (version 8.0).

## RESULTS

The sequential extraction of leaf, flower and fruit of *Vitex negundo* and bulb of *Allium sativum* demonstrated that the percentage of yield increased with increasing polarity of the solvents from DCM to aqueous for the leaf and fruit of *Vitex negundo* and bulb of *Allium sativum* but for the flower of *Vitex negundo* it increased up to methanol and then decreased to aqueous (Table 1).

Antibacterial screening of test samples on both *X.axonopodis* and *P.solanacearum* revealed the presence of inhibitive potentiality in all the test samples at various degrees. The inhibitory effect of these test samples differed significantly ($P<0.05$) and the diameter of inhibition zone for *X.axonopodis* and *P.solanacearum* ranged from 10.5 ± 0.7 to 42.4 ± 1.6 and 9.9 ± 0.5 to 48.5 ± 1.3 respectively (Table 2).

Among the test samples ethyl acetate, ethanol and methanol extracts of all plant parts showed better inhibition on test pathogens and the inhibitory effect was comparatively less in DCM and aqueous extracts (Table 2). Although ethyl acetate extract of flower and fruit of *Vitex negundo* and bulb of *Allium sativum* produced significantly ($P<0.05$) higher inhibition on both the test pathogens, ethyl acetate extract of flower showed the highest inhibition with the diameter of zone of inhibition of 42.4 ± 1.6 and 48.5 ± 1.3 on *X.axonopodis* and *P.solanacearum* respectively. Among the leaf samples ethyl acetate extract had significantly ($P<0.05$) higher

inhibition on *X.axonopodis* compared to other leaf extracts, however the effect did not differ significantly (P>0.05) with ethanol and methanol extracts of same plant part for the inhibition of *P.solanacearum* (Table 2).

**Table 1: The yield percentage of different parts of *V.negundo* and bulb of *A.sativum* in various solvents**

| Plant part | Extraction yield (%) | | | | |
|---|---|---|---|---|---|
| | DCM | Ethyl acetate | Ethanol | Methanol | Aqueous |
| ***V. negundo* Leaf** | 1.56 | 3.72 | 5.70 | 6.20 | 10.15 |
| ***V. negundo* Flower** | 0.53 | 0.62 | 3.23 | 6.43 | 5.48 |
| ***V. negundo* Fruit** | 2.11 | 5.36 | 6.44 | 6.74 | 8.62 |
| ***A. sativum* bulb** | 1.92 | 2.85 | 5.22 | 7.21 | 9.54 |

*DCM- Dichloromethane*

Among the test ethanol extracts, flower of *Vitex negundo* exhibited significantly (P<0.05) higher inhibition against both the test bacteria. Methanol extracts of fruit of *Vitex negundo* showed significantly (P<0.05) higher inhibition on *P.solanacearum* compared to other methanol extracts. Among the aqueous extracts, *Allium sativum* showed significantly (P<0.05) higher inhibition on *X.axonopodis*. DCM extract of fruit of *Vitex negundo* and *Allium sativum* also showed considerable inhibition on *P.solanacearum* (Table 2).

**Table 2: Antibacterial activity of different solvent extracts of *V, negundo* and *A. sativum***

| Test sample | | Diameter of inhibition zone (mm) * | |
|---|---|---|---|
| | | ***X.axonopodis*** | ***P.solanacearum*** |
| ***V.negundo* - leaf** | DCM | 11.1 ± 0.8 $^{nm}$ | 9.9 ± 0.5 $^{n}$ |
| | Ethyl acetate | 24.2 ± 1.0 $^{f}$ | 21.2 ± 1.0 $^{gh}$ |
| | Ethanol | 20.5 ± 0.4 $^{h}$ | 21.9 ± 0.7 $^{gf}$ |
| | Methanol | 19.1 ± 0.6 $^{ji}$ | 20.5 ± 1.4 $^{ih}$ |
| | Aqueous | 10.5 ± 0.7 $^{n}$ | 11.3 ± 0.3 $^{mn}$ |
| ***V.negundo* - fruit** | DCM | 10.7 ± 0.6 $^{nm}$ | 14.1 ± 0.4 $^{kj}$ |
| | Ethyl acetate | 38.6 ± 1.8 $^{b}$ | 34.0 ± 1.0 $^{b}$ |
| | Ethanol | 21.9 ± 0.3 $^{g}$ | 27.9 ± 0.8 $^{d}$ |
| | Methanol | 20.2 ± 0.7 $^{hi}$ | 23.1 ± 0.7 $^{f}$ |
| | Aqueous | 11.8 ± 0.7 $^{lm}$ | 14.2 ± 0.7 $^{j}$ |
| ***V.negundo* - flower** | DCM | 12.7 ± 0.9 $^{lk}$ | 12.2 ± 1.2 $^{ml}$ |
| | Ethyl acetate | 42.4 ± 1.6 $^{a}$ | 48.5 ± 1.3 $^{a}$ |
| | Ethanol | 31.6 ± 0.6 $^{d}$ | 32.0 ± 1.0 $^{c}$ |
| | Methanol | 22.0 ± 1.0 $^{g}$ | 19.2 ± 0.6 $^{i}$ |
| | Aqueous | 13.1 ± 0.4 $^{k}$ | 12.8 ± 0.9 $^{kl}$ |
| ***A. sativum* - bulb** | DCM | 11.2 ± 0.4 $^{nm}$ | 15.1 ± 0.9 $^{j}$ |
| | Ethyl acetate | 34.0 ± 1.0 $^{c}$ | 26.0 ± 0.7 $^{e}$ |
| | Ethanol | 26.5 ± 0.8 $^{e}$ | 21.9 ± 0.7 $^{gf}$ |
| | Methanol | 20.9 ± 0.9 $^{hg}$ | 19.7 ± 0.7 $^{i}$ |
| | Aqueous | 18.1 ± 0.8 $^{j}$ | 12.5 ± 0.5 $^{ml}$ |
| **Streptomycin (50 µg / 100 µl)** | | 22.1 ± 0.4 | 24.0 ± 0.4 |
| **Acetone : DMSO (1:1 v/v)** | | - | - |

*- No activity, * Zone of inhibition includes the diameter of well (8mm), Values are mean ± SD, Values with different superscript on the same column are significantly (P < 0.05) different. DCM- Dichloromethane, DMSO- Dimethylsulfoxide.*

The standard antibiotic streptomycin showed higher inhibitory effect on *P.solanacearum* than *X.axonopodis*. Further, The inhibition produced by the ethyl acetate extract of bulb *Allium sativum* and ethyl acetate and ethanol extracts of flower and fruit of *Vitex negundo* on *P.solanacearum* and ethyl acetate extracts of leaf and fruit of *Vitex negundo* and ethyl acetate

and ethanol extracts of flower of *Vitex negundo* and bulb of *Allium sativum* on *X.axonopodis* were comparatively higher than the standard on respective test bacteria. There was no inhibition exerted by the control (Table 2).

The MIC of selected test samples ranged between 2.5 mg / ml to 40 mg / ml (Table 3). Ethyl acetate extract of flower of *Vitex negundo* expressed lowest MIC value of 2.5 mg/ ml against both *X.axonopodis* and *P.solanacearum*. But it was 5 mg/ ml and 10 mg/ ml for ethyl acetate extract of fruit on respective pathogens.

Qualitative phytochemical analysis of the test samples revealed the presence of all the test phytochemicals in ethanol and methanol extracts of leaf and flower and methanol extract of fruit of *Vitex negundo*, whereas ethanol extract of fruit and all the ethyl acetate extracts of *Vitex negundo* possessed all the tested phytochemicals except saponins. In *Allium sativum*, ethanol, methanol and aqueous extracts possessed cardiac glycosides, terpenoids and saponins. Comparatively DCM extract possessed a very few number of phytochemicals tested (Table 4).

**Table 3: Minimum inhibitory concentration (MIC) of different solvent extracts of *V.negundo* and *A.sativum***

| Plant extract | Minimum inhibitory concentration (MIC) in mg/ml | |
|---|---|---|
| | *X.axonopodis* | *P.solanacearum* |
| Ethyl acetate extract of fruit of *V.negundo* | 5 | 10 |
| Ethyl acetate extract of flower of *V.negundo* | 2.5 | 2.5 |
| Ethanol extract of flower of *V.negundo* | 40 | 40 |
| Ethyl acetate extract of bulb *A.sativum* | 40 | 40 |

**Table 4: Phytochemical analysis of different solvent extracts of *V. negundo* and *A. sativum***

| Plant part | Extract | Al | Fl | Ta | Sa | C g | Te |
|---|---|---|---|---|---|---|---|
| *V.negundo* Leaf | DCM | + | - | - | - | + | - |
| | Ethyl acetate | + | + | + | - | + | + |
| | Ethanol | + | + | + | + | + | + |
| | Methanol | + | + | + | + | + | + |
| | Aqueous | + | + | - | + | + | - |
| *V.negundo* Fruit | DCM | - | - | - | - | + | - |
| | Ethyl acetate | + | + | + | - | + | + |
| | Ethanol | + | + | + | - | + | + |
| | Methanol | + | + | + | + | + | + |
| | Aqueous | - | - | - | + | - | - |
| *V.negundo* Flower | DCM | + | - | - | - | + | + |
| | Ethyl acetate | + | + | + | - | + | + |
| | Ethanol | + | + | + | + | + | + |
| | Methanol | + | + | + | + | + | + |
| | Aqueous | + | + | - | + | - | - |
| *A.sativum* Bulb | DCM | - | - | - | - | + | + |
| | Ethyl acetate | + | + | - | - | + | + |
| | Ethanol | - | - | - | + | + | + |
| | Methanol | + | - | - | + | + | + |
| | Aqueous | - | - | - | + | + | + |

*Al – Alkaloids, Fl – Flavonoids, Ta – Tannins, Sa – Saponins, Cg - Cardiac glycosides, Te- Terpenoids*

## DISCUSSION

The use of plant extracts is found to be an effective way of controlling plant diseases compared to synthetic chemicals as plant extracts have several advantages over it [12]. *Xanthomonas*

*axonopodis* pv. *citri* (formerly *Xanthomonas campestris* pv. *citri )* causes canker disease in all types of important citrus crops. Millions of dollars are spent annually on prevention, quarantines, eradication programs, and disease control in world wide [8, 13]. *Pseudomonas solanacearum* (Syn. *Ralstonia solanacearum*) causes bacterial wilt in a wide range of important crop plants including potato, tobacco, tomato and groundnut [9]. Control of these diseases requires integrated cultural practices and chemical sprays with copper compounds, but available measures are not effective and one of the major limitations of using chemical control agents is the development of resistance in bacteria [14]. In this study DCM, ethyl acetate, ethanol, methanol and aqueous extracts of leaf, flower and fruit of *Vitex negundo* and bulb of *Allium sativum* were subjected for the antibacterial activity against two phytopathogenic bacteria *Pseudomonas solanacearum* and *Xanthomonas axonopodis*. The data clearly revealed the pronounced activity of ethyl acetate extract of flower and fruit of *Vitex negundo* and bulb of *Allium sativum* and ethanol extract of flower of *Vitex negundo* against all the test bacteria (Table 2) and the MIC value was ranged from 2.5 mg / ml to 40 mg / ml. Phytochemical analysis of these four extracts revealed the presence of the alkaloids, flavonoids, tannins, cardiac glycosides and terpenoids. These bioactive molecules are reported to give resistance to plants against pests and pathogenic infections [15, 16].

It was also observed that the effect was higher in ethyl acetate extract and less in other four extracts. This indicates that the active constituents of the plant parts have more ability to dissolve in ethyl acetate solvent than other solvents used in this study. It has been already reported that the ethyl acetate extract of *Vitex agnus-castus* and *Vitex negundo* showed higher inhibitory effect on some other bacterial pathogens [17, 6].

Though aqueous and methanol produced higher amount of extract they exhibited relatively lower effect than ethyl acetate extract which was obtained in lower quantity. It indicates that the amount of yield does not always influence in inhibiting the growth of bacteria but the active ingredients found in the extract play a major role.

In the present study plant powder was sequentially extracted with different solvents in increasing polarity order. It was found that the polarity of the solvents seems to play an important role in the extraction of natural products which influences the antibacterial activity of the extracts [4] and in a sequential extraction technique, chemical constituents are partially separated according to their polarity, the least polar components separates into the low polar solvents and this progressing through the separation of active components based on their polarity and the polarity of the solvent used [18]. This partial separation of active components may be an advantage to reduce the antagonistic effects of chemical constituents because the compounds present in crude mixture may interfere with the action of the other [19].

Antibacterial properties of plant extracts against human pathogenic bacteria have been reported by several studies but only a few studies have been done on plant pathogens using plant extracts. It has been reported that aqueous extract of leaf of *Allium sativum* was found to have inhibitory effect on *Pseudomonas solanacearum* and *Xanthomonas campetris* [20]. It was also found that the aqueous extract of bulb of *Allium sativum* exhibited inhibition against *Xanthomonas campetris* pv *citri* [2]. But in the present study, though aqueous extract of bulb of *Allium sativum* showed inhibition on both test bacteria, ethyl acetate extract of *Allium sativum* exhibited significantly ($P<0.05$) higher inhibition than aqueous extract.

*Allium sativum* and *Vitex negundo* have been used as effective source for the treatment of several diseases in traditional medicine [3]. The results of present study support their inhibitory effect to

control the growth of phytopathogenic bacteria. To the best of our knowledge this is the first study for the antibacterial activity of *Vitex negundo* on *Pseudomonas solanacearum* and *Xanthomonas axonopodis* pv. *citri.* In order to identify the active antibacterial compounds from the extracts further investigation is needed. The work is being continued in our laboratory.

## CONCLUSION

This study revealed the antibacterial activity of different solvent extracts of leaf, flower and fruit of *Vitex negundo* and *Allium sativum* against *Pseudomonas solanacearum* and *Xanthomonas axonopodis* pv. *citri in vitro*. Among the test extracts ethyl acetate extract of flower and fruit of *Vitex negundo* and bulb of *Allium sativum* and ethanol extract of flower of *Vitex negundo* showed higher inhibitory effect. Further, this is the first report that demonstrates the inhibitory effect of *Vitex negundo* on *Pseudomonas solanacearum* and *Xanthomonas axonopodis* pv. c*itri*. These active extracts could be taken to the next step of bioassay guided purification to characterize the novel antibacterial agents.

## REFERENCES

[1] A. Rhouma, H. Ben Daoud, S. Ghanmi, H. Ben Salah, M. Romdhane, M. Demak, *Journal of Plant Pathology*, **2009**, 91, *2*, 339-345.
[2] M.A. Akhtar, M.H. Rahber-Bhatti, M. Aslam, *International Journal of Pest Management*, **1997**, 43, *2*, 149-153.
[3] D.M.A. Jayaweera, Medicinal plants (Indigenous and Exotic) used in Ceylon, National science council of Sri Lanka, Sri Lanka, **1981**.
[4] J. Parekh, S. Chanda, *Indian Journal of Pharmaceutical Sciences*, **2006**, 68, *6*, 835- 38.
[5] P. Josling, *Adv Ther.*, **2001**, 18, *4*, 189-93.
[6] S. Arokiyaraj, K. Perinbam, P. Agastian, R. Mohan Kumar, *International Journal of Green Pharmacy*, **2009**, 3, *2*, 162-64.
[7] H.O. Edeoga, B. Mbaebie, *African Journal of Biotechnology*, **2005**, 4, 685-688.
[8] OEPP/EPPO, *OEPP/EPPO Bulletin*, **2005**, 35,289–294.
[9] OEPP/EPPO, *OEPP/EPPO Bulletin*, **2004**, 34, 173 –178.
[10] Y. Vaghasiya, R. Nair, S. Chanda, *International Journal of Green Pharmacy*, **2009**, 3, *2*, 165-66.
[11] A. Sarkar, K.A. Kumar, N.K. Dutta, P. Chakraborty, S.G. Dastidar, *Indian Journal of Medical Microbiology*, **2003**, 21, *3*, 172-178.
[12] E.U. Opara, R.C. Wokocha, *Agricultural Journal*, **2008**, 3, *3*, 163- 170.
[13] A.K. Das, *J. Appl. Hort.*, **2003**, 5, *1*, 52-60.
[14] D.C. Sigee, Bacterial plant pathology: Cell and molecular aspects, Cambridge University Press, Cambridge **1993**.
[15] R.A. Dixon, *Nature*, **2001**, 411, 843-847.
[16] Y.H. Wang, M. Gueho, K. Hostettmann, *Phytochemistry*, **1989**, 28, 2323-2327.
[17] S.L. Khokra, O. Prakash, S. Jain, K.R. Aneja, D. Yogita, *Indian J. Pharm. Sci.*, **2008**, 70, *4*, 522-526.
[18] B.S. Furniss, A.J. Hannaford, V. Rogers, P.W. Smith, A.R. Tatchell, Vogel's Textbook of practical organic chemistry including qualitative organic analysis, Longman Inc., New York, **1978**.
[19] G.M. Adwan; B Ali Abu-Shanab; KM Adwan. *Pak. J. Med. Sci.*, **2008**, 24, *4*, 541-544.
[20] L.G. Lirio, M.L. Hermano, M.Q. Fontanilla, *Pharmaceutical Biology*, **1998**, 36, *5*, 357–359.