Agricultural Sciences, 1 (1996) 7-11

Antifungal Activity from Leaves of *Acacia Nilotica* against *Pythium Aphanidermatum*

A. J. Khan*, A. A. Zouba# and D. G. Seapy+

#Department of Plant Sciences, College of Agriculture, and +Department of Chemistry, College of Science, Sultan Qaboos University, P.O. Box 34, Al-Khod 123, Sultanate of Oman

خلاصة : تم تصنيف حامض الجاليك وإستر الميثيل لحامض الجاليك كمركبات مضادة للفطريات ضد النمو الفطري لفطر بايثم أفاندرماتم من مستخلصات الماء - الأسيتون لأوراق الهشاب. توقف نمو الفطر تماماً بتركيز ١٠٠٠ و ٧٥٠ جزء في المليون من حامض الجاليك وإستر الميثيل للحامض على التوالي. ووجد أن الخواص المضادة للفطريات كانت أكبر عند مزج المركبين معاً عن كل على حده، وكان أقل تركيز للمركبين مثبط للفطر هو ١٠٠ جزء في المليون . ولم تتم ملاحظة تأثير أي سميات نباتية للمركبات على نمو بذور البطيخ. بينما تأثر نمو جذور وسيقان بادرات البطيخ إيجاباً بحامض الجاليك وسلباً باستر الميثيل لحامض الجاليك. كلا المركبين كبتا بصورة معنوية نشاط أنزيم الفطر المختزل للنترات.

ABSTRACT: Gallic acid and methyl ester of gallic acid has been identified as antifungal compounds against the mycelial growth of *Pythium aphanidermatum* from acetone-water extracts of *Acacia nilotica* leaves. The growth of fungus was completely ceased by gallic acid and its methyl ester at 1000 ppm and 750 ppm, respectively. Antifungal properties of both compounds were found to be higher in combination than alone. The minimum inhibitory concentration for both compounds was 1000 ppm. No phytotoxic effect of the compounds was observed on watermelon seed germination. The growth of roots and shoots of watermelon seedlings was promoted by gallic acid but decreased with methyl ester of gallic acid. Nitrate reductase activity of the fungus was significantly inhibited by both compounds.

Gum tree, *Acacia nilotica* (Linn.) Delile, is one of the most important tanniferous plant. Tannins are a very important group of natural compounds with great structural diversity and wide polygenic distribution. They are particulary important as raw material to tan hides and for several other industrial uses (Devi and Prasad, 1991). The pods of *A. nilotica* have been used in tanning and in folklore medicine in diarrhoea, gynaecological diseases, haemorrhage, colds and ophthalmia (Watt and Breyer-Brandwijk, 1962). The fruit and bark juices can also be applied to syphilitic and mouth lesions. Tannins are known to possess antimicrobial (Janardhanan et al. 1963) and molluscicidal properties (Khalid et al. 1989). A considerable number of reports show that the pods, heartwood and bark of *A. nilotica* contain various tannins including gallic acid and gallic acid methyl ester (Khalid et al., 1989 ; Adewoye and Rao, 1977; Ishak, 1974). However, no reports are available on the presence of gallic acid and its methyl ester in the leaves of *A. nilotica*.

The extensive application of synthetic fungicides has been hindered due to their phytotoxicity, carcinogenicity and residual effects. Some plant products have been reported to possess natural antifungal activity against the phytopathogenic fungi (Tripathi, 1977). These products have high biological activity, little or no phytotoxicity and are degradable (Fawcett and Spencer, 1970).

Pythium diseases, such as seed rot and damping-off are among the most common soil borne diseases causing serious destruction world wide of vegetable crops, grasses, fruit trees and ornamental plants (Hendrix and Campbell, 1973). Currently, diseases caused by *Pythium* sp. are minimized by seed treatment and the use of systemic fungicides (Cohen and Coffey, 1986). However, one of the most commonly used broad spectrum group of fungicides, ethylene bisdithiocarbamate, has been suggested to cause tumours in mice and rats (Ulland, 1972). The more recently introduced systemic fungicides, such as metalaxyl and Fosetyl-Al have been shown to generate resistance to some isolates of *Pythium aphanidermatum* (Sanders et al., 1990). Similarly, Deahl and Inglis (1995) have isolated metalaxyl-insensitive strain of *Phytophthora infestans* from infected tubers of *Solanum sarachoides* in Northwestern Washington.

The present investigation was designed to ascertain the antifungal activity of *Acacia nilotica* leaves as an alternative disease control treatment against *Pythium aphanidermatum*. Isolation and identification of the active compounds from *A. nilotica* leaves and its fungitoxic properties were determined.

* Corresponding Author

Materials and Methods

Leaves of *Acacia nilotica* L. were collected from the Botanic garden of Sultan Qaboos University. Fresh leaves (500 g) were ground in a high speed blender 2-3 times with dichloromethane to remove majority of lipids and chlorophyll and filtered. Debris was exhaustively extracted with acetone-water mixture (60:40; v/v) and extracts were combined (Porter, 1989). Acetone was evaporated *in vacuo* at < 40 °C and the residue was diluted with distilled water and stored at 4 °C in the dark for further studies. The extract was tested for its antifungal activity against *Pythium* sp. The fungus was isolated from the roots of diseased watermelon and identified by Centraalbureau Voor Schimmelcultures, Baarn, The Netherlands as *Pythium aphanidermatum*. An aqueous extract was mixed with potato dextrose agar (PDA) medium in concentration of 40, 80, 160, and 240 mg fresh weight/ml of medium. Mycelial disc of 4 mm diameter were inoculated in the centre of petri dishes containing PDA supplemented with leaf extract and plates were incubated at room temperature. Growth inhibition of the fungus was recorded at 24, 48, and 72h incubation periods.

The concentrated acetone-water extract (80g) was subjected to column chromatography over Sephadex LH-20 (Pharmacia Biotech. AB, Sweden). The column (4.5 x 60 cm) was eluted with ethanol at a flow rate of 1 ml/min and 15 ml fractions were collected. Fractions were monitored by silica gel TLC using chloroform-ethyl formate-formic acid (3:6:1) solvent system, and identical fractions were pooled together yielding 5 compounds **1** (109 mg), **2** (189 mg), **3** (1.20 g), **4** (1.89 g), and **5** (2.00 g). The antifungal activity of these compounds was tested against *P. aphanidermatum* using the paper disc method as described by Tripathi and Dixit (1977). The assay discs (13 mm in diameter, Whatman # 1) were prepared by impregnating the compound dissolved in ethanol and evaporating the solvent with hot air. Discs prepared similarly with the same volume of ethanol were used as control. One assay disc was transferred to the centre of each petri dish containing PDA medium. Mycelial discs, 4 mm diameter, were cut from the periphery of 24 h old culture and placed upside down in the centre of each assay disc in treatment and control sets. Colony diameters were measured after 24 and 48 h. Further purification of the most active compounds **1** and **2** was achieved by rechromatography on Sephadex LH-20 eluting with ethanol - water (1: 3) to give compound **1** (80 mg) methyl ester of gallic acid, an off-white powder, UVλ nm 283; ^{1}H-NMR δ (CD_3 OD): 3.79 (3H, OCH_3), 7.04 (2H, H-2/6); ^{13}C-NMR δ (acetone-d_6): 121.5, 110.1, 146.3, 139.8, 169.0 (C=O), 52 (OCH_3); compound **2** (112 mg) gallic acid, UVλmax 267; ^{1}H-NMR δ (CD_3 OD) 6.55 (s, Ar-H); ^{13}C-NMR δ ($CDCl_3$) 167.72, 144.41, 137.07, 120.54, 108.79. The IR spectra (KBr pellet) of compound **1** and **2** were identical to the IR spectrum of gallic acid methyl ester and gallic acid, respectively (Pouchert, 1981). Both compounds were also identified by TLC using authentic samples of gallic acid and gallic acid methyl ester.

Fungitoxic effect of the purified compounds was carried out separately and in combination by using a paper disc assay method described elsewhere (Tripathi and Dixit, 1977) on watermelon pathogen, *P. aphanidermatum*.

Healthy watermelon seeds were used for seed germination test. They were soaked for 24 hr in gallic acid and methyl easter of gallic acid at concentrations of 500, 1000, 1500 & 2000 ppm. The seeds were then blot dried and 30 seeds were placed on a moist filter paper in a petri dish. Observations on seed germination, root and shoot length and leaf emergence were made after 72 h.

The effect of gallic acid and its methyl ester on nitrate reductase activity of *P. aphanidermatum* was also studied. For *in vivo* studies, fungus was grown in Glucose Asparagine liquid medium supplemented with 500 ppm of gallic acid or gallic acid methyl ester. Mycelial biomass was harvested at regular intervals. For nitrate reductase enzyme 500 mg mycelium in 1 ml of 0.1 M Tris buffer pH 7.0 was ground with fine carborundum powder in a pre - chilled mortar and pestle. Homogenate was centrifuged at 20,000 g for 15 minutes and the volume of supernatant made up to 10 ml with the buffer (Mahadevan and Sridhar, 1986). The enzyme reaction mixture contained 1 ml of enzyme extract, 1 ml of 0.1 M KNO_3 , 1 ml of 0.1 mM NADH (ß-nicotinamide adenine dinucleotide reduced form) and 1 ml of potassium phosphate buffer, pH 7.0. The reaction mixture was incubated for 20 min at 30 °C and reaction was stopped by adding 1 ml of 1% sulphanilamide in 1.5 N HCL and 1 ml of 0.02% N-(1-naphthyl) ethylenediamine hydrochloride. Blanks were maintained without NADH. The absorbance of pink colour produced was measured at 540 nm in a colorimeter. The amount of nitrite produced was calculated from standard curve obtained by plotting different conc. of $NaNO_2$ against their respective optical densities. For *in vitro,* fungus was grown in liquid medium for 5 days without any supplement. Enzyme reaction mixture was supplemented with gallic acid or gallic acid methyl ester to give final concentrations of 125, 250, 500, 750 and 1000 ppm. After 1 h incubation, production of nitrite was estimated as described above. All experiments were conducted in a minimum of three replicates and repeated twice unless otherwise mentioned.

Results and Discussion

The results showed that the acetone-water extract of *Acacia nilotica* wt possessed strong antifungal activity against *Pythium aphanidermatum* (Table 1). The growth of the fungus was greatly reduced at 80 mg/ml concentration, whereas growth completely ceased at 160 mg fresh wt/ml of medium after 24 and 48 h incubation periods. Extracts of tannin bearing plants have been reported as effective antibacterial, antifungal and virucidal (Janardhanan et al. 1963, Khan et al., 1993). Leaf decoctions from several plants including *A. nilotica*, were found by Rahber-Bhatti (1988) to be inhibitory to the leaf rust of wheat. Fungitoxic effect of compounds **1**, **2**, **3**, **4**, and **5** are shown in Table 2. The mycelial growth of the fungus was substantially inhibited at 600 ppm by compounds **1** and **2**, whereas **3**, **4**, and **5** were less effective after 48 h. Compounds **1** and **2** were rechromatographed on Sephadex LH-20 yielding pure gallic acid methyl ester and gallic acid, respectively. When tested on mycelial growth by paper disc assay method, both compounds showed significant control on the growth. After 48 h fungal growth was ceased at 750 ppm by gallic acid methyl ester and 1000 ppm by gallic acid (Table 3). However, when both compounds were mixed in the ratio of 1:1 and tested against fungal growth, the effect was more inhibitory. The growth of the test organism completely ceased at 750 ppm (Table 4). The tannin contents of *A. nilotica* have been studied in detail and several reports indicate the presence of gallic acid and gallic acid methyl ester in pods and bark (Khalid et al., 1989; Adewoye and Rao, 1977 and Ishak, 1974), but no report seems to be available on the presence of these compounds in leaves. As far as authors are aware, this is the first time that the antifungal activity of *A. nilotica* has been studied in detail. Aqueous or alcoholic extracts of leaves and other parts of the plant have been tested for fungitoxic properties against several phytopathogenic fungi (Rawat et al., 1992; Tripathi and Dixit, 1977; Tripathi et al. 1978 and Tripathi et al., 1980). Recently, Daayf et al. 1995 have reported that aqueous extracts from leaves of knotweed (*Reynoutria sachalinensis*), when applied weekly at a concentration of 2%, provided control of powdery mildew on English cucumber that was as effective as benomyl. No report is available for the identification of antifungal compound from leaves of *A. nilotica*. However, Sastry et al. (1963) have reported epi-Gallocatechin from the ethyl acetate soluble fraction of the bark from *A. arabica*, showing fungitoxicity against rice fungus, *Piricularia oryzae*. This seems to be the first report on the identification of gallic acid and gallic acid methyl ester as an antifungal agent from leaves of *A. nilotica*.

Effect of gallic acid and gallic acid methyl ester on the seed germination of watermelon showed that both compounds are not phytotoxic up to 1500 ppm concentration, however, slight pytotoxicity (40% to 42%) was evident at 2000 ppm. A significant increase in root length was noticed with gallic acid and little or no increase in shoot length was observed at 1000 ppm and above (Figure 1), whereas gallic acid methyl ester was observed to be inhibitory for root and shoot growth.

As nitrate reductase is the most important and rate limiting enzyme in the assimilation of exogenous nitrate (Tripathi, 1977), its regulation by gallic acid and gallic acid methyl ester was therefore studied in *Pytium aphanidermatum*. In the control, the enzyme activity was increased reaching an optimum at day 5 and declined in older cultures. Both compounds at 500 ppm inhibited the enzyme activity at different incubation periods (Figure 2). The maximum decline in enzyme

TABLE 1

Effect of crude acetone-water extract of Acacia nilotica leaves on mycelial growth of Pythium aphanidermatum (data are average of three replicates).

Incubation Period (h)	% Inhibition of Mycelial Growth Aqueous Extract (mg fresh wt/ml medium)			
	40	80	160	240
24	47.2	78.9	100.0	100.0
48	5.5	72.1	100.0	100.0

TABLE 2

Fungitoxic effect of different concentrations of partially purified compounds (1, 2, 3, 4 & 5) from leaves of Acacia nilotica on mycelial growth of Pythium aphanidermatum (data are average of three replicates).

Incubation Period (h)	Concentration (ppm)	% Inhibition of Mycelial Growth Compounds				
		1	**2**	**3**	**4**	**5**
24	300	56.8	47.3	8.8	24.4	4.9
	600	100.0	100.0	17.8	55.1	7.3
48	300	50.9	45.0	2.7	9.4	0.5
	600	84.7	82.4	40.5	51.8	26.7

TABLE 3

Effect of different concentrations of gallic acid and methyl ester of gallic acid on mycelial growth of Pythium aphanidermatum. Data are average of three replicates.

Incubation Period (h)	Compound	% Inhibition of Mycelial Growth 250 ppm	500 ppm	750 ppm	1000 ppm
24	Gallic acid	11.6	57.7	70.5	100.0
	Gallic acid methyl ester	15.4	82.6	100.0	100.0
48	Gallic acid	3.9	49.2	70.8	100.0
	Gallic acid methyl ester	25.6	59.4	100.0	100.0

TABLE 4

Effect of different concentrations of gallic acid and gallic acid methyl ester combined together on mycelial growth of Pythium aphanidermatum (data are average of three replicates).

Incubation Period (h)	% Inhibition of Mycelial Growth Final Concentration (ppm)*		
	250	500	750
24	53.8	100.0	100.0
48	57.5	81.9	100.0

* Final concentration is a mixture of both compounds in 1:1 ratio.

TABLE 5

Evaluation of phytotoxic effect of gallic acid and gallic cid methyl ester on watermelon seed germination (data are the average of three replicates).

Concentration (ppm)	% Inhibition	
	Gallic Acid	Gallic Acid Methyl Ester
0	36	36
500	32	36
1000	28	31
1500	37	24
2000	40	42

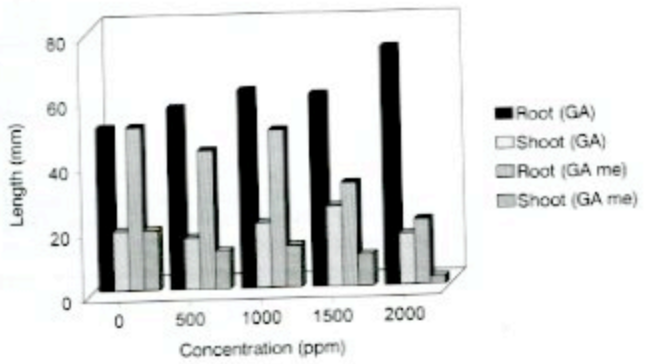

Figure 1. Phytotoxic effect of gallic acid (GA) and gallic acid methyl ester (GA Me) on root and shoot growth of watermelon seedlings. Data are the average of three replicates.

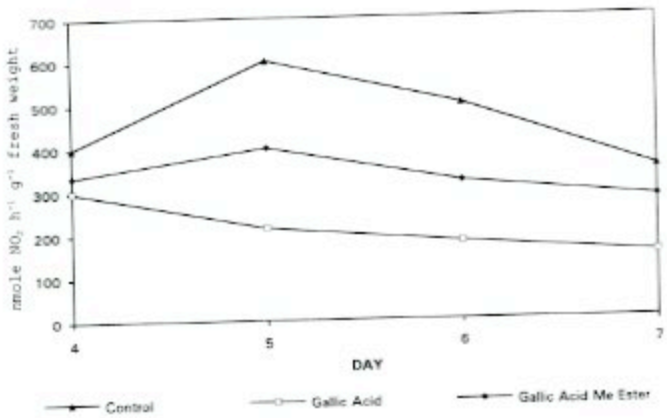

Figure 2. Effect of gallic acid and gallic acid methyl ester (500 ppm) on in vivo nitrate reductase activity of Pythium aphanidermatum. Enzyme activity is expressed as nmole NO_2 h^{-1} g^{-1} fresh weight of fungal mycelium.

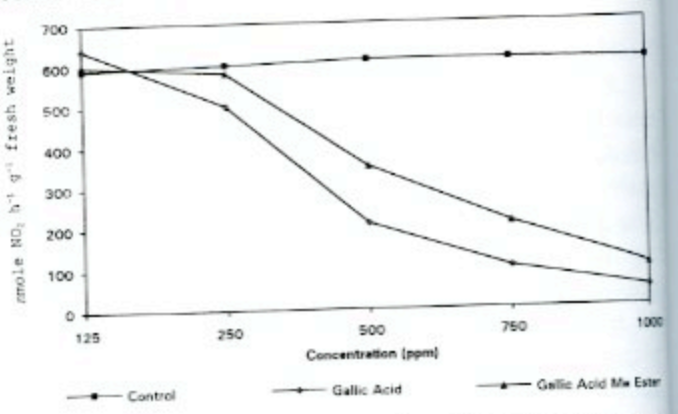

Figure 3. Effect of different concentration of gallic acid and gallic acid methyl ester after 5 days of incubation period on in vitro nitrate reductase activity of Pythium aphanidermatum. Enzyme activity is expressed as n mole NO_2 h^{-1} g^{-1} fresh weight of fungal mycelium.

activity with 500 ppm of gallic acid was noticed at 64% as compared to the control after 5 and 6 days, whereas, maximum inhibition (36%) was found with gallic acid methyl ester after 6 days of incubation period. Inhibition of nitrate reductase increased with increase in the concentration of gallic acid methyl ester and gallic acid. Enzyme activity was slightly stimulated (8.5%) with gallic acid and not with its methyl ester at 125 ppm concentration but decreased after that reaching a maximum inhibition of 95.0% and 83.5% with 1000 ppm of gallic acid and gallic acid methyl ester, respectively (Figure 3). However, the inhibitory effect of gallic acid *in vivo* and *in vitro* was more than gallic acid methyl ester. Regulation of nitrate reductase and its properties have been extensively investigated in fungal system (Cove 1966; Lewis and Fincham, 1970 and Choudhary and Rao, 1973). Effects of antibiotics on fungal nitrate reductase enzyme have been demonstrated earlier (Gottlieb and Shaw, 1970). In the present investigation, it is evident that gallic acid methyl ester and gallic acid both inhibited the nitrate reductase enzyme.

Conclusions

It can be concluded from the present work that the leaves of *Acacia nilotica* are a potent source of control for *Pythium aphanidermatum*. It may be mixed with soil and used as possible control for damping-off diseases of vegetables. Investigations leading to *in vivo* control using leaves of *A. niolotica* need to be done.

Acknowledgement

The authors wish to acknowledge the assistance of Dr. Pradeep Tripathi, Scientist, National Chemical Laboratory, Pune, India for NMR data. The help of Dr. Ahmed K. Al-Rawahy, Mubarak Al-Oraimy and Yusuf Al-Maqbaly is gratefully acknowledged.

References

Adewoye, R.O. and J.B. Rao. 1977. *Acacia nilotica*, variety adonsonii pods (Bagaruwa) of Nigeria - Part II. *Leather Science* 24:293-301.

Choudhary, V.P. and G. Rao. 1973. Induction of nitrate reductase in the yeast, *Candida utiles*.Annual Meeting of Association of Microbiologist of India.

Cohen, Y. and M.D. Coffey. 1986. Systemic fungicides and the controlof oomycetes. *Ann. Rev. Phytopathol.*, 24:311-338.

Cove, D.J. 1966. The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. *Biochem. Biophys. Acta* 113:51-56.

Daayf, F., A. Schmitt and R.R. Belanger. 1995. The effects of plant extracts of *Reynoutria sachalinensis* on powdery mildew development and leaf physiology of long English cucumber. *Plant Disease*, 79(6):577-580.

Deahl, K.L. and D.A. Inglis. 1995. Occurrence of metalaxyl-insensitive *Phytophthora infestans* on *Solanum sarachoides* in Northwestern Washington. *Plant Disease* 79(5): 540.

Devi, S.R. and M.N.V. Prasad. 1991. Tannins and related polyphenols from ten common *Acacia* species of India. *Bioresource Technol.*, 36:189-192.

Fawcett, C.H. and D.M. Spencer. 1970. Plant chemotherapy with natural products. *Ann. Rev. Phytopathol.*, 8:403-418.

Gottlieb, D. and P.D. Shaw. 1970. Mechanism of action of antifungal antibiotics. *Ann. Rev. Phytopathol.*, 8:371-402.

Hendrix, F.F. Jr. and W.A. Campbell. 1973. Pythiums as plant pat hogens. *Ann. Rev. Phytopathol.*, 11:77-98.

Ishak, M.S. 1974. Some aspects on the chemistry of phlobaphenes from *Acacia nilotica* bark. *Egypt. J. Chem.*, 17:699-703.

Janardhanan, K.K., D. Ganguly, J.N. Baruah and P.R. Rao. 1963. Fungitoxicity of extracts from tannin-bearing plants. *Current Science* 5:226-227.

Khalid, S.A., S.M. Yagi, P. Khristova and H. Duddeck. 1989. (+)-Catechin-5-galloyl ester as a novel natural polyphenol from the bark of *Acacia nilotica* of Sudanese origin. *Planta Medica* 55:556-558.

Khan, A.J., A. Zouba and M.O. Al-Matrushi. 1993. Control of *Pythiumaphanidermatum* by extracts of *Acacia* sp. *Phytopathology* 83:174 (abst.).

Lewis, C.M. and J.R.S. Fincham. 1970. Regulation of nitrate reductase in the basidiomycetes, *Ustilago maydis*. *J. Bacteriol.*, 103:55-61.

Mahadevan, A. and R. Sridhar. 1986. *Methods in Physiological Plant Pathology (3rd Edition)*. Sivakami Publication, Madras, India.

Porter, L.J. 1989. Tannins. In: *Methods in Plant Biochemistry, volume I*, J.B. Horbone (Editor) 389-419. Academic Press, N.Y.

Pouchert, C.J. (Editor). 1981. *The Aldrich Library of Infrared Spectra (3rd Edition)*. Aldrich Chemical Company, Milwaukee, WI.

Rahber-Bhatti, M.H. 1988. Antifungal properties of plant leaf dec octions against leaf rust of wheat. *Pakistan J. Bot.*, 20:259-263.

Rawat, A.K.S., S. Mehrotra and U. Shome. 1992. Antimicrobial activity of *Thalictrum foliolosum*.*Fitoterapia* 63:545-546.

Sanders, P.L., M.D. Coffey, G.D. Greer and M.D. Suika. 1990. Laboratory induced resistance to fosetyl-al in a metalaxyl resistance field isolate of *Pythium aphanidermatum*. *Plant Disease* 74:690-692.

Sastry, G.P., J.N. Baruah and P.R. Rao. 1963. (-)-epi-Gallocatechin from *Acacia arabica* bark. *Indian J. Chem.*,1:542-543.

Tripathi, R.D. 1977. Assay of higher plants for antifungal antibiotics and some aspects of mode of action of the active principle. Ph.D. Thesis, Gorakhpur University, India.

Tripathi, S.C. and S.N. Dixit. 1977. Fungitoxic properties of *Rosa chinensis* Jacqub. *Experientia* 33:207.

Tripathi, R.D., H.S. Srivastava and S.N. Dixit. 1978. A fungitoxic principle from the leaves of *Lawsonia inermis* Lam. *Experientia* 34:51-52.

Tripathi, R.D., S.C. Tripathi and S.N. Dixit. 1980. Structure activity relationship amongst some fungitoxic α-naphthoquinones of angiosperm origin. *Agric. Biol. Chem.*, 44:2483-2485.

Ulland, B.M. 1972. Thyroid cancer in rats from ethylenethiourea intake. *J. Natl. Cancer Inst.*, 49:583-584.

Watt, J.M. and M.G. Breyer-Brandwijk. 1962. *Medicinal and Poisonous Plant of Southern and Eastern Africa (2nd Edition)*. 546 E.S. Livingstone Ltd., Edinburgh and London.

Published with the approval of the College of Agriculture, S.Q.U., as paper number 130895.