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Activity of extracts from tropical and sub-tropical spices and herbs against plant pathogenic fungi

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Abstract
The antifungal activity of ethanolic extracts of spices and herbs were studied *in vitro* and *in vivo* experiments. In *in vitro* screenings extracts (1% m/v) from *Mentha piperita*, C*oriandrum sativum*, *Piper nigrum*, *Carum carvi* and *Urtica dioica* were found with high activity against conidial germination of *Cladosporium cucumerinum*, *Botrytis cinerea* and *Alternaria solani* and additionally of *P. nigrum* and *U. dioica* which inhibited efficiently mycelium growth of *C. cucumerinum* and *A. solani*. *In vivo* extracts of *P. nigrum*, *C. carvi*, *U. dioica*, *Cassia* spp. and *F. vulgare* showed significant levels of disease protection activities against *A. solani*, *Phytophthora infestans* and *Oidium lycopersicum* on tomatoes, *Erysiphe graminis* f. sp. *tritici* on wheat and *Uromyces appendiculatus* on beans. Active compounds from the seed extract of *P. nigrum* were isolated and characterised with TLC and HPLC analysis. The study enabled that extracts from spices and herbs are sources which could assist environmentally safe ways in the management of fungal pathogens.

Introduction
Natural products with biological activities against insects, plants and plant pathogenic microorganisms have provided inspirations in agrochemistry to look for new products in managing crop pests and diseases (Jespers and Waard 1993). Nicotine from extracts of tobacco leaf to control lace bug, rotenone from roots of derris and pyrethrum from flower heads of *Chrysanthemum cinerariaefolium* are pioneer insecticides of plant origin (McCallan 1969). Plant extracts solely or as secondary metabolites are among the modern tools in sustainable, safe and environmentally sound ways of plant protection being used as fungicides or inductors of resistance. An aqueous extract of the medicinal plant *Rheynoutria sachalinensis* (*Polygonaceae*) showed protectant effects to fungal diseases like powdery mildews with acting as inducer of resistance in the host plants (Herger et al. 1988). Traditionally, spices are used as preservatives of foods and agricultural products against insect pests and disease damages in the tropics and sub-tropics (Secoy and Smith, 1983). The works of Gorris and Smid (1995) and Wilson et al. (1996) also showed substances from spice extracts in securing quality and quantity of food products. In contrast there are only scarce knowledge for the use of these plant extracts in controlling of plant pathogenic fungi. The objective of this study was the screening of

spices and herbal extracts for their potential as sources of antifungal substances under *in vitro* and *in vivo* conditions and to isolate and characterize their biochemical nature.

Materials and Methods

Plants
Lycoperisicon lycopersicum (L.) Farw. cv. 'Rheinlands Ruhm'; *Capsicum annum* L. cv.'Yolo wondor B'; *Triticum aestivum*, L. cv. 'Kanzler, Toronto'; *Phaseolus vulgaris* L. cv. 'Saxa'; *Vitis vinifera* L. cv. 'Müller-Thurgau' were used. Plants were raised either by sowing or cuttings. During the growing periods, plants were supplied with 16 hours artificial light (approx. 7000 lx), 60 to 80 % RH, 20 ± 2°C, twice irrigation per day and 2 % standard nutrient solution for every week.

Fungi
Isolates of *Botrytis cinerea* (Pers. ex. Fr.), *Cladosporium cucumerinum* Ellis & Arth., *Alternaria solani* ((Ellis et Martin) Sorauer), *Phytophthora infestans* (Mont.) de Bary, *Plasmopara viticola* (Berk. et M.A. Curtis) Berl. et de Toni in Sacc.), *Oidium lycopersicum* (Cooke et Masse), *Uncinula necator* (Schw.) Burr) and *Erysiphe graminis* DC. f. sp. *tritici* (Em. Marchal), *Uromyces appendiculatus* ((Pers.) Unger) and *Puccinia recondita* (Rob. ex Desm.) were used. Culturing and maintenance of the first three fungi were performed on Potato-Dextrose-Agar (PDA) at 20 ± 5°C. *P. infestans* was cultured on vegetable juice-calcium carbonate agar (200ml V_8 juice; 3 g $CaCO_3$; 16 g agar; 800 ml a. dest.) at 18°C in the dark. Conidia of the powdery mildews were regularly inoculated to healthy plants under glasshouse conditions to keep up survival. The downy mildew was preserved on detached leaves bearing fully matured sporangiospores at −18 °C. Rust fungi were cultured on susceptible plants and their urediospores were stored below -18°C.

Spices and herbs
The commercially available spices and herbs were investigated: *Mentha piperita* L. (Labiatae); *Origanum vulgare* L.(Labiatae); *Anethum graveolens* L. (Umbelliferae); *Ocimum basilicum* L. (Labiatae); *Cassia* spp. (Caesalpiniaceae); *Cinnamomum verum* J.S.Presl (Lauraceae); *Elettaria cardamomum* (L.) Maton (Zingiberaceae); *Cuminum cyminum* L. (Umbelliferae); *Piper nigrum* L. (Piperaceae); *Carum carvi* L. (Umbelliferae); *Menyanthes trifoliata* L. (Menyanthaceae); *Thymus vulgaris* L. (Labiatae); *Brassica nigra* (L.) Koch (Cruciferae); *Urtica dioica* L. (Urticaceae); *Coriandrum sativum* L. (Umbelliferae); *Artemisia dracunculus* L. (Asteraceae); *Syzygium aromaticum* (L.) Merr. et L.M. Perry (Myrtaceae); *Majorana hortensis* Moench (Labiatae); *Petroselinum crispum* (Mill.) Nym. (Umbelliferae); *Pimenta officinalis* Lindl. (Myrtaceae); *Vernonia* spp. (Asteraceae) and *Foeniculum vulgare* Mill. (Umbelliferae).

Extraction of spices and herbs
Samples were grounded and 10 g of each were mixed in 100 ml ethanol (70 %) to harvest 10 % dry weight by volume ratio of an extract. Samples were extracted at 60 °C for 2 hours described by Blaeser and Steiner (1999) and Mekuria et al. (1999). The pellet and supernatants were separated by hydraulic filtration and the supernatant was stored at 4 °C.

Screening for antifungal activity in vitro
Inhibition of conidial germination was tested by amending PDA with the extracts. 5ml of each extracts was mixed into 100 ml sterile nutrient media. Each petri-dish was inoculated by seeding 10 ml spore suspension of the fungi, *B. cinerea* (5x 10^4 conidia /ml), *A. solani* (3x 10^4 conidia/ml), and *C. cucumerinum* (5x 10^4 conidia/ml). Media contained 5 ml of 70 % ethanol served as standard checks. In all cases, microscopically counts of spores, germinated and non germinated, were taken after one to three days of incubation at 20 ± 5°C. Extracts of *P. nigrum* and *U. dioica* (at 0.25, 0.5 and 1.0 % mv^{-1}) were mixed with PDA in petri-dishes, agar discs with one-week-old mycelium of the test fungi were placed onto the centre of each petri-dishes which then were incubated at room temperature. As standards, untreated or with 70 % ethanol treated agar plates were accommodated.

Bioassays for disease protection activity in vivo
Experiments on beans were conducted on fully-grown two cotyledon leaves and on wheat seedlings with fully-grown primary and secondary leaves. The remaining test plants attained the four leaf stages. *B. cinerea* (1×10^6 conidia/ml) on green pepper, *A. solani* (5×10^4 conidia/ml) on tomatoes; *P. infestans* (8×10^4 zoospores/ml) on tomatoes; *O. lycoperisicum* on tomatoes, *P. viticola* (7×10^4 sporangia/ml) on grape vine; *P. recondita* (9×10^4 urediospores/ml with 0.1% TWEEN 20 in 100 ml H_2O) on wheat; and *U. appendiculatus* (9×10^4 urediospores/ml with 0.1% TWEEN 20 in 100 ml H_2O) on beans were applied with a hand sprayer;. the inoculation of *E. graminis* on wheat and *U. necator* on grape vine were carried out by dusting of vital conidia from infected plants to new plants. Treatments were either incubated in a moist chamber at 20 to 25°C and 95 to 100 % RH or in glass houses at 20 to 25°C.

Thin-layer (TLC) and high performance liquid (HPLC) chromatographie
TLC plates as stationary phase (20 x 20 cm, Silica gel 60, F_{254}-precoated plate, Merck, Germany) and toluene-ethyl acetate (50:50) as mobile phase were employed to detect characteristic compounds in *P. nigrum* seed extract according to Wagner and Bladt (1997). Developed plates were used for detection (at 254 and 366 nm) of spore germination inhibitory fractionation and comparison with standards (piperine 97 % pure, Sigma-Aldrich Germany). 20 ml of spore suspensions of *A. solani* (2.5 x10^3 conidia/ml) were mixed with 40 ml of autoclaved PDA, sprayed onto the developed TLC plates and incubated for 3 days in a moist chamber. The major active fractions of the extract on TLC plates were recovered and re-suspended with acetonitrile-water (80:20) solvents for analysis by HPLC (HP 1050, Software HP Chem. 3.21) on a reverse phase column (Hilbar® pre-packed RT 125-4 Lichrosorb®RP-18, Merck Darmstadt, Germany). The diode array detector (DAD) was adjusted at 210, 230 and 270 nm of wave length. Samples were adjusted at the flow rates of 0.75 ml/min, at 40 °C and 400 bar in the following solvent gradient profiles: acetonitrile/water proportion (%) and time of resolution (min) 5/95(2), 10/90(4), 20/80(4), 40/60(4),60/40(4), 80/20(4), 100/0(4), 5/95(4).The volume of injection was varied from 5 to 25 µl.

Sampling, data collection and statistical analysis
Disease severity parameters were gathered as percentage of infected leaf areas, number of pustules or pustule sizes. Totally, 4 leaves of green pepper, tomatoes (bearing 12 leaflets per replication) and grape vine per replication were sampled for collecting of disease measurements. Disease status from bean plants were recorded on two cotyledon leaves per replication. Five primary leaves per replication were considered to have data on

powdery mildews of wheat. Efficacy was computed by adopting the methods of Abbott (1925). Curve fittings for dose-response interrelationships, estimation of ED_{50} values and sensitivity parameters (β) have been achieved by using a model of Ortega et al. (1997). Analysis of variance and multiple separations of means (Tukey- and t-tests) was determined by using Sigma Stat, Jandel Scientific Inc. (SPSS, Version 2, 1997, USA).

Results and Discussion

Inhibition of conidial germination by spice and herbal extracts

The *in vitro* screening of extracts from spices and herbs against spore germination of *A. solani*, *B. cinerea* and *C. cucumerinum* indicated differences in their antifungal activity (fig. 1). More than half of the tested materials showed either no or very low spore germination inhibitory characteristics, but 25 to 35 % of the tested spices and herbal extracts impaired the germination of conidia efficiently. Similarly, fungal spore germination inhibitory effects of plant extracts from A*llium sativum* (Bianchi et al. 1997) and essential oils from spice extracts (Zambonelli et al. 1996) have been reported. Spores or conidia represent important stages for the survival of plant pathogenic fungi. Development of strategies impairing the germination and germ-tube formation of spores by use of substances from spices and herbal extracts implicit the high potential of reduction of spread and epidemic development of fungal pathogens.

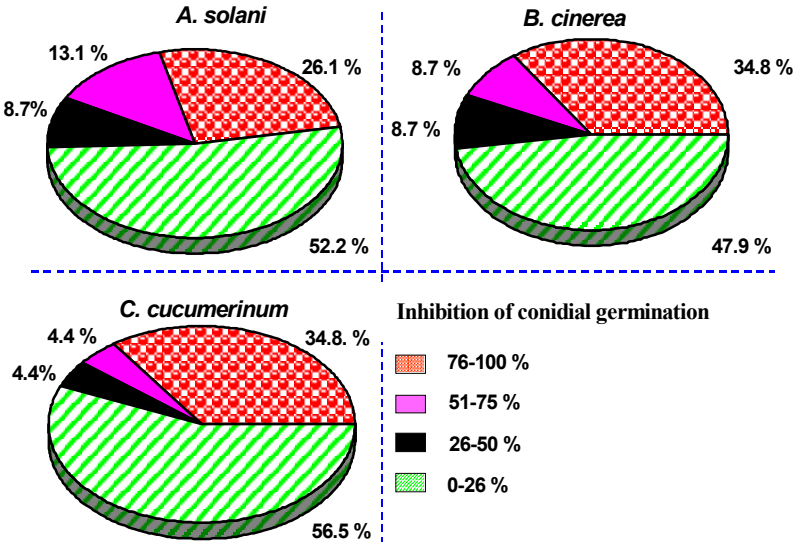

Figure 1: Frequency of ethanolic extracts from 23 tropical and subtropical spices and herbs showing different ranges in inhibition of the conidial germination of plant pathogenic fungi in *in vitro* tests.

Inhibition of mycelium growth by spice and herbal extracts

The extracts of *P. nigrum* and *U. dioica* impaired *in vitro* the mycelium growth of *A. solani* (fig. 2). The activity of the extracts was strongly correlated to the applied dosages. Compared to the normal growth of *A. solani* growth restriction effects exceeded 95 % at high concentration. This was accompanied by deformation and crumbling in radial growth of the mycelium. Substances showing spore germination inhibitory activity might not express similar inherent mode-of-action effects on other life stages of test fungi. However, screening of products for their impacts across the different life stages of fungi can provide some more information regarding the spectrum of biological activity, persistency and stability (Sisler 1977).

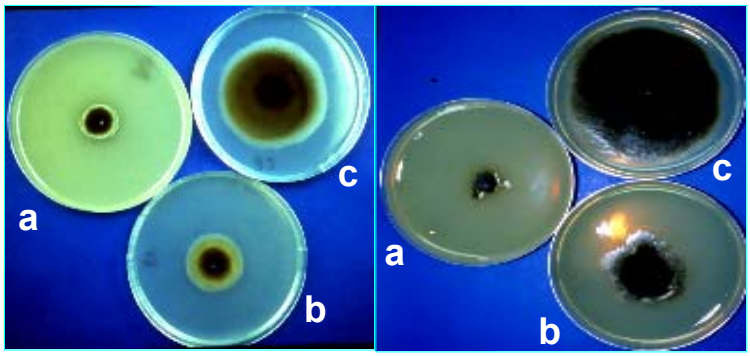

Figure 2: Antifungal effects of ethanolic extracts from *Piper nigrum* (left) and *Urtica dioica* (right) on mycelium growth of *Alternaria solani* on PDA. (concentrations: a = 1%, b = 0,5%, c = 0,25%).

Effects of spice and herbal extracts against fungal pathogens on plants

The efficiency of the extracts of spices and herbs in plant protection in different host pathogen interactions is shown in table 1.

Table 1: Antifungal effects of ethanolic extracts of spices and herbs on disease intensity of different plant pathogenic fungi on plants in the greenhouse.

Botanical name	efficiency in reduction of disease symptoms (%)*			
	Alternaria solani [1]	*Phytophthora infestans* [1]	*Oidium lycopersicum* [1]	*Erysiphe graminis* [2]
disease severity of untreated plants	100 %	61.9 %	24.2 pustules/leaf	41.0 %
M. piperita	+	++	±	++
O. vulgare	±	++	+	+
A. graveolens	±	±	+	+
O. basilicum	±	++	++	±
Cassia spp.	±	++	+++	+++
C. verum	±	+	++	+
E. cardamomum	±	±	±	+
C. cyminum	±	+	±	+
P. nigrum	+++	+++	+++	++
C. carvi	+	+++	+++	+++
M. trifoliata	+	++	±	±
T. vulgaris	±	±	±	±
B. nigra	±	±	±	+
U. dioica	±	+++	++	++
C. sativum	++	+++	+	++
A. dracunculus	+	+++	++	++
S. aromaticum	+	±	±	+
M. hortensis	±	+	±	±
P. crispum	±	+	+	++
P. officinalis	±	±	±	+
F. vulgare	++	++	++	++
Vernonia spp.	+	+	±	+

*efficacy : +++ = very high (76-100 %); ++ = moderate (51-75 %); + = low (26-50 %); ± = very low (25 %)

1 = host plant: tomatoes, 2= host plant: wheat seedlings

Results depicting only extracts from *P. nigrum*, *C. carvi*, *Cassia* spp, *A. dracunculus*, *U. dioica, F. vulgare*, and *C. sativum* could frequently show significant levels of disease reduction. Similar to the result of the *in vitro* testing the activity of pepper extracts was in most cases superior to the other treatments. Likewise, herbal extracts from *P. erecta* and *S. officinalis* (Blaeser and Steiner 1999) as well as extracts from bryophytes have been reported with high levels of *in vitro* and *in vivo* antifungal bioactivities (Mekuria et al. 1999).

Mode-of-action of the seed extract of P. nigrum
Black pepper extracts exhibited both protective and curative mode-of-actions against *P. recondita* of wheat (fig.3). Application of the extract 120, 48, and 4 hours before inoculation and 24 hours past inoculation reduced rust pustules as compared to the rust severity on untreated plants and curative treatments 72 and 120 hours past inoculation. Applications at least 4 hours before inoculation resulted in a protection of the plants higher than 95%.

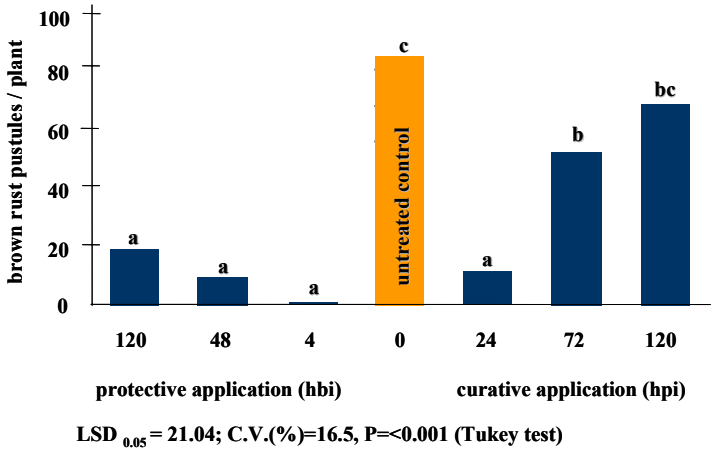

Figure 3: The influence of the time interval between protective and curative application of the ethanolic seed extract of *Piper nigrum* and inoculation on the disease intensity of *Puccinia recondita* on wheat.

Activity spectrum of the seed extract of P. nigrum
The extract showed a wide range of antifungal activity impairing infection densitiy of plant pathogens differing in their mode of parasitism. High levels of efficacy (> 90 %) were recorded against bean and wheat rusts, followed by effects on early blight, late blight, downy mildew and gray mould. The ED_{50} –values (% v/v) and high levels of sensitivity parameters imply that strong fungicidal effects of pepper extracts on the tested fungi occurred (tab. 2)

Characterization of antifungal compounds from seed extracts of P. nigrum
TLC-analysis of pepper seed extracts demonstrated six inherent constituents as shown in figure 5 (I), which are numerated from solvent fronts to solvent bases. The major activity ($\mathbf{f_4}$) was retained at Rf-value of 0.5. White bands on TLC plate implicate zones of inhibition in conidial germination of *A. solani*. Detection of all the bands under UV-light at 366 and 254 nm excited distinct fluorescence and that of $\mathbf{f_4}$ showed similar nature of ignitions like standard amides control utilised. Piperetin, piperanin, piperaestin, piperyline and piperine from *P. nigrum* extracts have been reported by Wagner and Bladt (1997) with the aid of TLC analysis and Ahmed et al. (1975) with colorimetric methods. They noted that piperine constitute 4 to 10 % of the total plant extract. Comparison of $\mathbf{f_4}$, the crude extract and this standard molecule in

Table 2: Intrinsic sensitivity and fifty percent dose levels (ED_{50} % v/v) for disease reduction of the seed extracts of *P. nigrum* in different host pathogen systems.

pathogen	host plant	ED $_{50}$ (% v/v)	sensitivity parameter (β)	coefficient of determination (%)
B. cinerea	green pepper	0.425	0.495	99.7 **
A. solani	tomatoes	0.260	0.461	80.6 *
P. infestans	tomatoes	0.530	0.444	91.8 **
P. viticola	grape vine	0.968	0.576	98.6 **
O. lycopersicum	tomatoes	0.899	0.238	96.7 **
U. necator	grape vine	1.320	0.221	65.8 *
E. graminis	wheat	0.651	0.215	82.9 *
U. appendiculatus	bean	0.250	0.665	95.1 **

* with significant correlation values,
** with highly significant correlation values

HPLC analysis showed common features in studied spectral ranges. Co-injection of the standard with **f_4** resulted in an increase in the peak height, but did not affect basic retention time. Application of piperine as biotic fungal disease protectant has not been reported and shows distinct bioactivity as opposed to piperidin, which has been widely utilised as an active ingredient for insecticides.

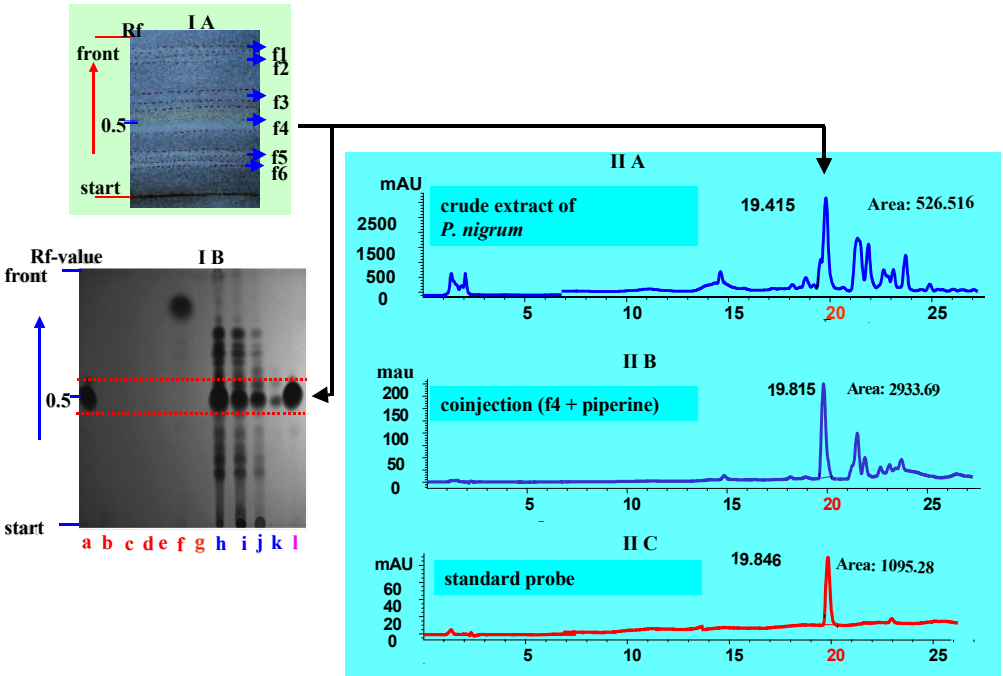

Figure 5: Bioautographic and chromatographic of the ethanolic seed extract of *Piper nigrum* illustrating characterisation of major active fraction (f_4). IA= TLC bioautographic overlay assay; IB = comparision of f4 with standard probes under UV-light (254 nm) [standards a = piperine, b = piperidin, c = 1,3-pentadien, d = limonen, e = pinen, f = eugenol, g = caryophyllene, crude extract and fractionation h = dichloromethan extract, i = methanol extract, j = ethanol extract, k = f_4 reisolated from TLC and coinjection l = f_4 + piperine]HPLC resolution time of active ingredient profiles of the crude extract (IIA) , coinjection (IIB) and standard sample (IIC) at 210 nm (DAD).

Conclusion

The study confirms that the widely known bioactive nature of spice and herbal extracts can be used as sources of compounds with antifungal activity. The effects might be derived from single molecule, derivatives of molecules or the crude extracts themselves. The findings of only a small number of spices with interesting fungicidal effects reflect that future works should focus on screening a large range of diverse plant extracts to harvest target molecules. However, it still demands large scale production and field experimental works before onset of any practical applications.

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