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Biological Activity of Peanut (*Arachis hypogaea*) Phytoalexins and Selected Natural and Synthetic Stilbenoids

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Abstract

The peanut plant (*Arachis hypogaea* L.), when infected by a microbial pathogen, is capable of producing stilbene-derived compounds that are considered antifungal phytoalexins. In addition, the potential health benefits of other stilbenoids from peanuts, including resveratrol and pterostilbene, have been acknowledged by several investigators. Despite considerable progress in peanut research, relatively little is known about the biological activity of the stilbenoid phytoalexins. This study investigated the activities of some of these compounds in a broad spectrum of biological assays. Since peanut stilbenoids appear to play roles in plant defense mechanisms, they were evaluated for their effects on economically important plant pathogenic fungi of the genera *Colletotrichum*, *Botrytis*, *Fusarium*, and *Phomopsis*. We further investigated these peanut phytoalexins, together with some related natural and synthetic stilbenoids (a total of 24 compounds) in a panel of bioassays to determine their anti-inflammatory, cytotoxic, and antioxidant activities in mammalian cells. Several of these compounds were also evaluated as mammalian opioid receptor competitive antagonists. Assays for adult mosquito and larvae toxicity were also performed. The results of these studies reveal that peanut stilbenoids, as well as related natural and synthetic stilbene derivatives, display a diverse range of biological activities.

Keywords

Arachis hypogaea; peanuts; groundnuts; stilbenoids; resveratrol; phytoalexins; biological activity; antifungal; antitumor; anticancer; cytotoxic; antioxidant; anti-inflamatory; opioid receptor; adult mosquito; mosquito larvae

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INTRODUCTION

Stilbene-derived compounds are commonly present in plants and have become of great interest to many research groups worldwide because of the diverse range of biological activities that they tend to display (1,2). When infected by a microbial pathogen, the peanut plant (*Arachis hypogaea*) becomes a potent producer of a distinctive set of stilbene-derived phytoalexins, (3–5). Peanut stilbenoids have been considered the major sustaining factor of the plant's resistance to diseases (3). In addition, the health benefits of resveratrol (**24**) (Figure 1) from peanuts and other plants have been established by many investigators (1,2,6).

Peanut stilbenoids are polyphenolic compounds that are isolated from peanut plant materials strictly in the *trans* olefinic configuration. The *trans*-olefin structure of the parent stilbene skeleton is an important determinant of bioactivity (1). The major stilbenoids bear isopentenyl, isopentyl, or isopentadienyl moieties arising from prenylation (3–5). Prenylation plays a major role in the diversification of many natural aromatic compounds, including those from peanuts. For example, many prenylated flavonoids have been identified as constituents in plants, and display biological activities, such as anti-cancer, anti-androgen, anti-*Leishmania*, and anti-nitric oxide production (7). Prenylation also provides greater lipophilicity to molecules, allowing them to readily penetrate through cell membranes. An increase in lipophilicity (commonly expressed as a log P constant) often correlates positively with increased biological activity within groups of compounds of similar structure (8).

As reported by several researchers (8,9–12), the number and positions of hydroxy groups in stilbenoids tend to be crucial factors in their biological activities. These groups may serve as essential, regulating, or enhancing moieties in such compounds (8,10–13). For example, a 4′-hydroxy group was demonstrated to be essential for the cytogenetic and estrogenic activities of stilbenoids (1,11). The higher reactivity of compounds with a 4′-hydroxy group compared to those having 3′- and 5′-hydroxy groups can be attributed to resonance effects (1).

Other aspects of the structure-activity relationship in substituted stilbenes have been investigated. The olefinic double bond in the stilbene skeleton was found to be necessary for tyrosinase inhibitory activity (14). The stilbene moiety also displayed higher antifungal activity against *Gloeophyllum trabeum* and *Poria placenta* compared to the corresponding bibenzyl moiety within a group of otherwise structurally similar compounds (8,10,13). Similar results were later reported for the inhibition of *Botrytis cinerea* (15).

While the bioactivity of *trans*-resveratrol has been extensively studied (1,2,6), the effects of prenylated peanut stilbenoids have been only minimally explored (16–19). Anticancer properties of selected peanut stilbenoids, arachidin-1 (**1**), arachidin-3 (**3**), *trans*-3′-isopentadienyl-3,5,4′-trihydroxystilbene, and resveratrol (**24**) were investigated (19); compopunds **1**, **3**, and **24** showed concentration-dependent growth inhibitory effects on HL-60 cells. Arachidin-1 (**1**) appreciably induced mitochondrion-mediated apoptosis at low concentrations and was demonstrated to be an effective anticancer agent that was capable of inducing caspase-independent death of cancer cell with mutations in apoptotic genes (19). These data indicate the need for screening of recently discovered peanut stilbenoids (4,5) for anticancer and/or other beneficial or adverse effects on other organisms. Modern medicine still demands intensive research on health-beneficial properties of natural stilbenoids and their synthetic analogs (1,2,12). Publications (18,20) on the bioproduction and isolation of **1**, **3**, and **24** from hairy root cultures of the peanut plant encourage *in vivo* study of peanut stilbenoids.

The purpose of this investigation was to study biological activities of important natural peanut stilbenoids and compare their biological activities to those of some natural stilbenoids from different plant sources as well as selected synthetic stilbene derivatives. Evaluation of antifungal, anti-inflammatory, cytotoxic, and antioxidant activities was performed. In addition, mosquito adult and larval mortality assays were conducted, and the affinity of these stilbenoids to opioid receptors was examined.

MATERIALS AND METHODS

General experimental procedures

HPLC-grade solvents used in the preparation of mobile phases were obtained from Fisher (Suwanee, GA). HPLC-grade H_2O was prepared with a ZD20 four-bowl Milli-Q water system (Millipore). Deuterium oxide (99.9 atom% D) as well as HPLC-grade benzene (99.8%), toluene (99.8%), ethylbenzene (99.8%), propylbenzene (98%), and butylbenzene (99+%) were purchased from Sigma-Aldrich. All chemicals used for opioid receptor tests were also from Sigma-Aldrich with the following exceptions. For the binding experiments, [3H]DAMGO (53.4 Ci/mmol), [3H]U-69,593 (42.7 Ci/mmol), [3H]Enkephalin (45 Ci/mmol), were obtained from Perkin-Elmer Life Sciences Inc. (Boston, MA). DAMGO ([D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin), DPDPE ([D-Pen^{2,5}]-enkephalin), and nor-binaltorphimine were obtained from Tocris Bioscience (Ellisville, Missouri).

Tested compounds

Peanut phytoalexins **1**–**10** were obtained as described (4,5,21). Compounds **11**–**21** (Figure 1) were a gift from Dr. T.P. Schultz (Department of Forest Products, Mississippi State University, MS). Chlorophorin (**22**) was prepared as described (22). Reference samples of rhapontin (**23**) and resveratrol (**24**) were purchased from Sigma-Aldrich. Identities and purity of all compounds were confirmed by means of UV, NMR and mass spectrometry (4,5,10,13). MS analysis of individual compounds tested did not reveal any unrepresentative mass units and therefore the compounds were evaluated as “MS-pure.” Chromatographic purity of all tested compounds was >98.0% based on HPLC area percent measurements that were performed at the light absorption maxima for each individual compound.

HPLC-DAD-MS analyses

Analysis of compounds tested was performed using a tandem HPLC-MS Surveyor HPLC system equipped with MS Pump Plus, Autosampler Plus, a PDA Plus Detector (Thermo Electron Corp., San Jose, CA), and a 50 mm × 3.0 mm i.d., 2.5-μm, XBridge C18 analytical column. H_2O (A), MeOH (B), and 2%-HCOOH in H_2O (C) were mixed in the following gradient: initial conditions, 68% A/ 30% B/ 2% C, changed linearly to 0% A/ 98% B/ 2% C in 12 min, held isocratic for 5 min, then changed to initial conditions in 0.01 min. The flow rate was 0.5 mL/min. The column was maintained at 40° C in a model 105 column heater (Timberline Instruments, Boulder, CO).

Mass Spectrometric measurements

ESI-MS/MS^n data were obtained on a Finnigan LCQ Advantage MAX ion trap mass spectrometer equipped with APCI interface and operated with Xcalibur™ version 1.4 software (Thermo Electron Corporation, San Jose, CA). All data were acquired in the full-scan positive and negative polarity modes from *m/z* 100 to 2000. Capillary temp. was 270° C, sheath gas flow 60 units, auxiliary/sweep gas flow 20 units, source voltage 4.5 kV. In MS^2 analyses, the $[M+H]^+$ and $[M-H]^-$ ions observed for each chromatographic peak in full-scan analyses were isolated and subjected to source collision-induced dissociation (CID) using He buffer gas. In all CID analyses, the isolation width, relative fragmentation energy,

relative activation Q, and activation time were *m/z* = 2.4, 35–40%, 0.25, and 30 ms, respectively.

Estimation of octanol-water partition constants

Octanol-water partition constants (log P or K_{OW}) of all tested compounds were deduced using an HPLC method. HPLC equipment and solvents were the same as listed above. A Gemini C_{18} analytical column (5-μm particle size, 4.6 × 150 mm; Phenomenex) and an isocratic mobile phase composed of MeOH, H_2O and 2% HCOOH in H_2O (55:43:2, w/w) were used for the experiments. The flow rate was 1.0 mL/min and the column temperature was 37 °C. Before injecting into the system, the compounds were dissolved in the mobile phase. At least 5 injections of standards and tested compounds were performed. The following homologous compounds with known log P constants (given in parentheses) were used as standards: benzene (2.13), toluene (2.73), ethylbenzene (3.15), propylbenzene (3.69), and butylbenzene (4.26) (23,24). A linear regression that was used for calculation of log P values was determined using the SigmaStat software, version 3.5 (Systat Software, Inc., Chicago, IL) and was characterized by high $R^2 = 0.995$. Capacity factor, K, was calculated based on the equation, $K = (t_1 - t_0) / t_0$, where t_1 is the retention time of the tested compound, and t_0 is the void volume of the analytical column. The void volume of the column was determined by injecting unretained neat D_2O into the HPLC system.

Antifungal assay

A modified method of the National Committee for Clinical Laboratory Standards (NCCLS) M27-A reference method for broth dilution antifungal susceptibility testing of yeast was adapted (25) for evaluation of antifungal compounds against agriculturally important conidia-forming filamentous fungal plant pathogens to natural compounds in comparison to known fungicidal standards. The method (25) was used to evaluate the antifungal activity of test compounds towards *Botrytis cinerea, Colletotrichum acutatum, C. fragariae, C. gloeosporioides, Phomopsisviticola, P. obscurans*, and *Fusarium oxysporum.* The experimental procedures and the sources of fungal isolates were described (25). The commercial fungicide captan was used as an internal fungicide standard in all assays. The SAS system analysis of variance procedure (Statistical Analysis System, Cary, NC) was used to identify significant factors, and Fisher's protected LSD was used to separate means.

Cytotoxicity assay

Cytotoxicity was determined against a panel of four human tumor cell lines [SK-MEL (malignant melanoma); KB (oral epidermal carcinoma); BT-549 (breast ductal carcinoma); and SK-OV-3 (ovary carcinoma)] ; and two noncancerous cell lines [Vero (African green monkey kidney fibroblasts) and LLC-PK_{11} (pig kidney epithelial cells)] as described earlier (26). Doxorubicin was used as a positive control.

Anti-inflammatory assays

Anti-inflammatory activity was determined in terms of the inhibition of NF-κB -mediated transcription and inhibition of intracellular generation of reactive oxygen species (ROS) and nitric oxide (NO). Inhibition of NF-κB mediated transcription was determined in human chondrosarcoma (SW1353) cells by a reporter gene assay as described earlier (28). Sp-1 was used as a control transcription factor to evaluate the toxicity of tested compounds in the same assay. Parthenolide was used as the positive control. Inhibition of intracellular NO production as a result of iNOS activity was assayed in mouse macrophages (RAW 264.7 cells) as described (29). Parthenolide was included in each assay as the positive control. Inhibition of intracellular ROS generation (antioxidant activity) was assayed in human

promyelocytic leukemia (HL-60) cells by using DCFH-DA as described previously (30). Trolox was used as a positive control.

Cell culture for opioid receptor assay

Cho-K1 cells stably transfected with opioid receptor subtypes μ, Δ, and κ were a generous gift from Dr. Brian Roth (University of North Carolina at Chapel Hill, Chapel Hill, NC). These cells were maintained at 37 °C and 5% CO_2 in a DMEM nutrient mixture supplemented with 2mM L-glutamine, 10% fetal bovine serum, 0.5% penicillin–streptomycin, and either G418 (600 mg/mL) or hygromycin B (300 mg/mL). Membranes were prepared by scraping the cells in a 50 mM Tris buffer, homogenized via sonication and centrifuged for 40 min at 13650 rpm at 4 °C. These were kept at −80 °C. Protein concentration was found via Bio-Rad Protein Assay (Hercules, CA).

Radio-ligand binding for opioid receptor subtypes

Opioid binding took place under the following conditions: 10 μM of each compound was incubated with [^{3}H]DAMGO (μ), [^{3}H]U-69,593 (κ), or [^{3}H]enkephalin (Δ) for 60 min in a 96-well plate. Tritium and membrane concentration for each cell line was determined by saturation experiments performed after each batch of membrane was scraped. The reaction was terminated via rapid vacuum filtration through GF/B filters presoaked with 0.3% BSA using a Perkin Elmer 96-well Unifilter followed by 10 washes with 50 mM Tris. Plates were read using a Perkin Elmer Topcount. Total binding was defined as binding in the presence of 0.1% DMSO. Non-specific binding was defined as binding observed in the presence of 10μM DAMGO (μ), nor-binaltorphimine (κ), or DPDPE (Δ). Specific binding was the difference between total and non-specific binding. Percent binding was found with the following formula: 100-(binding of compound- non-specific binding) × 100 / specific binding. Compound GC-143-8 was run in competition binding against all three opioid subtypes (μ, κ, and Δ). In short, concentrations of compound ranging from 100 μM to 48 nM were incubated for 60 min in a 96-well plate with a predetermined amount of [^{3}H] specific to each membrane type. Optimal membrane concentration was also predetermined by a saturation experiment. The reaction was terminated via rapid vacuum filtration through GF/B filters presoaked in 0.3% BSA using a Perkin Elmer 96-well Unifilter followed by 10 washes of 50 mM Tris. Plates were read using a Perkin Elmer Topcount. Total binding was defined as binding in the presence of 0.1% DMSO. Non-specific binding was defined as binding observed in the presence of 10μM CP55,940. Specific binding was the difference between total and non-specific binding. K_i and IC_{50} values were calculated using GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

Mosquito larvae and adult mosquito assays

Larval bioassays were performed as described (31). For assays against mosquito adults, stock chemical solutions prepared as described (31), were diluted into acetone to a final concentration of 6.25 μg/μL. Ten adult *A. aegypti* female mosquitoes, 3–5 d post-eclosion, were cold-anaesthetized and placed on a BioQuip chill table (Rancho Dominguez, CA) set at 4°C. One-half of a microliter (0.5 μL) of the test chemical was applied to the dorsal thorax of each insect using a #1702 gas-tight Hamilton syringe mounted onto a Hamilton PB600 repeating dispenser (Reno, NV), with a final dose of 3.12 μg per insect. For any chemical producing 50% or greater mortality, a second assay was performed using 1.56 μg per insect. After treatment, mosquitoes were placed in 3.5-oz plastic cups, containing 10% sucrose solution, and maintained at 28°C and 80% relative humidity. All mosquito larvae and adult mosquito assays were performed in triplicate. Controls included negative (untreated), carrier (DMSO-acetone), and positive (permethrin).

RESULTS AND DISCUSSION

Evaluation of antifungal activity

Since peanut phytoalexins appear to play a role in plant defense mechanisms (3), the stilbenoids (Figure 1) were evaluated first for their antifungal effects against plant pathogenic fungi. The fungal species tested are economically important worldwide (25) for many crops, including peanut. Botrytis blight of peanuts is caused by *Botrytis cinerea. Phomopsis* spp. frequently produce fruiting structures in the peanut necrotic tissue of leaf scorch lesions (secondary invasion of leaf tissue). *Phomopsis* spp. were commonly associated with *Colletotrichum* spp. in marginal necrotic peanut leaf and stem lesions. Several *Colletotrichum* spp. have been reported as casual agents of anthracnose on peanut (32). *Fusarium oxysporum* together with *F. solani* generally are the causal agents of Fusarium wilt of peanuts; these species also can dominate the pod microflora. Many *Fusarium* spp. that are normally soil saprophytes can become pathogenic to peanuts.

In order to possess biological activity, compounds must be able to move through cell membranes. The octanol/water partition constant (log P) is an important physical parameter that characterizes the lipophilicity of individual compounds. Log P values for all tested compounds were estimated using an HPLC method, which was characterized by low error values (Figure 1).

A standard procedure (25) was chosen for the antifungal study, so that the results obtained for the stilbenoids could be directly compared to those of other compounds that were tested using the same method. Lipophilicity–structure–antifungal activity relationships were estimated in a series of assays with significant fungi of the genera *Colletotrichum, Botrytis, Fusarium*, and *Phomopsis* (25). Compounds tested were found to be active only against three species: *Phomopsis obscurans*, *P. viticola*, and *Botrytis cinerea* (Figure 2). Chiricanine A (**5**), arahypin-1 (**6**), and arahypin-5 (**10**), with log P values 3.46, 3.71, and 3.34, respectively, when tested against *P. viticola* and *P. obscurans* after 144 h of incubation, were more fungicidal than compounds with lower (< 3) log P values (Figures 1 and 2A-C). However, no reliable lipophilicity–bioactivity correlation was deduced with compounds **11**–**24** (Figures 1 and 2D-G). For example, **23** (log P = 0.74) demonstrated activity similar to that of **22** (log P = 3.29), when assayed against *B. cinerea* at 72 h (Figure 2G). Apparently, the means by which compounds tested inhibit fungal growth cannot necessarily be directly linked to stilbenoid lipophilicity.

The importance of the olefinic double bond in bioactivity of such compounds has been recognized (8,10,13,15). To investigate whether some of tested compounds follow the same pattern observed with other such compounds, we tested two pairs of compounds, the stilbenoids **13** and **14**, and their bibenzyl analogues **17** and **16**, respectively. Surprisingly, no significant difference in the activity of these compounds was observed in any of the experiments. Moreover, **16** demonstrated statistically higher activity compared to **14** against *P. viticola* at 144 h at the highest tested concentration of 30 μM. Schultz et al (8,10) investigated the activity of **13** and **17**, and **14** and **16**, respectively, among other stilbenoids against *Coriolus versicolor*, *Gloeophyllum trabeum*, and *Poria placenta*. The results were mixed for **14** and **16** and were not significantly different. At the same time, **13** was significantly more active against *G. trabeum*, and *P. placenta* compared with **17**. The inhibitory effect of pinosylvin (**11**) was significantly greater than that of dihydropinosylvin (15). In contrast to our data, these results indicate the importance of the olefinic double bond for the activity against *G. trabeum*, and *P. placenta*.

The overall activity of peanut phytoalexins (Figure 1), particularly at lower concentrations, against *P. viticola* (Figure 2A) was higher in comparison with the activity of compounds **11**–

24 (Figure 2D). The antifungal properties of **5**, **6**, and **10** were similar to those of the standard, captan (Figure 2A) at 144 h. Captan is a multisite inhibitor fungicide with no systemic activity and is commonly used as an internal standard. The antifungal activity of all tested peanut stilbene derivatives at 120 h virtually matched their bioactivity at 144 h. Among compounds **11**–**24**, chlorophorin (**22**), **13**, **16**, and **19** (Figure 2D) demonstrated appreciable activity at the highest concentrations, while resveratrol (**24**) as well as **11** and **12** were essentially inactive.

On the other hand, stronger inhibition of *P. obscurans* was evident with compounds **11**–**24** (Figure 2E) relative to the natural peanut metabolites (Figure 2B-C). The majority of the compounds (Figure 2E), with the exception of **11**, **12**, **18**, and **24**, exhibited high selective activity at 30 μM against *P. obscurans* at 144 h. Three of the peanut metabolites, **5**, **6**, and **7** (Figure 1) demonstrated the highest levels of inhibition from 120 to 144 h. While the activity of **6** within this period of time statistically remained at the same level, the activity of **5** changed from low to very high, and the activity of **7** changed from very high to extremely low (Figure 2B-C). Since the other compounds retained their activity within the assay time, and since there was no sign of compound precipitation, this phenomenon could probably be attributed to an inducible detoxification mechanism in the fungal test strain. Interestingly, compounds **1**, **2**, **3**, and **4**, the stilbenoids that are often found in fungal-challenged peanuts at significantly higher levels than other stilbenoids (5), did not exhibit any activity against *P. obscurans* at 120 and 144 h (Figure 2B-C).

All tested peanut metabolites were inactive against the *Botrytis cinerea* strain at the levels tested. It is important to note that *B. cinerea* is an important pathogen of peanut, and there are no peanut cultivars known to be resistant to it(32). *B. cinerea* may be capable of effectively detoxifying the plant's stilbene-derived phytoalexins, or the fungus might send a signal to the host that does not allow to recognize the pathogen invasion.

In contrast to peanut stilbenoids, the majority of compounds **11**–**23** exhibited selective activity against the same *B. cinerea* strain (Figure 2F-G). Chlorophorin (**22**), at 48 and 72 h, and rhapontin (**23**), at 72 h, demonstrated very high growth inhibition values comparable with those of captan. Resveratrol (**24**), as well as **11** and **12**, showed minimal activity at 48 h, but showed no activity after 72 h of incubation, and actually appear to have promoted growth of *B. cinerea* (Figure 2F-G). The lack of influence of **24** on germination and growth of *P. viticola* and *B. cinerea* has been previously reported (33). In our experiments, pterostilbene (**21**), a dimethylated analogue of resveratrol (**24**), exhibited significantly stronger fungal growth-inhibiting properties than resveratrol (Figure 2D-G). Similar results have been published for the bioactivity of these compounds against *B. cinerea* (9).

In all the assays for fungal growth inhibition, neither **11**, **12**, nor **24** showed appreciable signs of activity (Figure 2D-G). The inactivity of resveratrol (**24**) may indicate a role in fungal challenged peanut seeds as a precursor of more active prenylated stilbenoids (4). Stilbene derivatives **11**–**24** (with the exception of **20** and **23**) were previously studied in assays against the wood-decaying fungi, *C. versicolor*, *G. trabeum*, and *P. placenta* (8, 10). Although the results cannot be compared directly with our data, we note that, in both studies, resveratrol (**24**) demonstrated negligible activity against the tested fungal species.

Analysis of the antifungal activity of the limited number of compounds **11**–**24** did not lead to conclusions regarding their structure-activity relationship that could help to explain the bioactivity of natural peanut stilbene derivatives. However, this research revealed that the new peanut metabolites **4**, **5**, **6**–**10** are capable inhibitors of *P. viticola* and/or *P. obscurans* (Figure 2A-C), and that some simpler stilbene derivatives also possess strong fungal growth inhibition properties (Figure 2D-G) against economically important fungi.

Evaluation of anti-inflammatory, antioxidant and cytotoxic activities

We further investigated biological activity of peanut phytoalexins together with some natural and synthetic stilbenoids in a panel of target oriented bioassays to explore their anti-inflammatory and antioxidant effects in mammalian cells (Table 1). The anti-cell proliferative activity was also evaluated in a panel of cell lines to determine their cytotoxic potential.

NF-*κ*B is a protein complex that controls the transcription of DNA. NF-*κ*B is found in animal cells and is involved in cellular responses to various exogenous and endogenous stimuli. Abnormal activation of NF-*κ*B is frequently observed in many cancers; suppression of NF-*κ*B limits the proliferation of cancer cells. NF-*κ*B also is an active participant in the inflammatory response. Therefore, compounds inhibiting NF-*κ*B signaling have potential therapeutic application in treatment of cancer and inflammatory diseases.

Peanut stilbenoids **1**, **3**, and **24**, and non-peanut stilbenoids, **11**, **13**, **19**, and **22** (Figure 1) demonstrated inhibition of NF-*κ*B-dependent transcription in SW1353 cells induced by phorbol myristate acetate (PMA) with IC_{50} values in the range of 12 – 22.5 μg/mL, as shown in Table 1. Unexpectedly, arachidin-2 (**2**) inhibited both NF-kB and Sp-1 at significantly lower concentrations (0.025 and < 0.025 μg/mL, respectively). These results are indicative of extremely high cytotoxicity or general nonspecific inhibition of transcriptional activity of **2** in this assay. Stilbenoids **11**, **13**, and **19** inhibited both NF-*κ*B and Sp-1 to a similar extent indicating that inhibition of NF-*κ*B was not specific and the effect could be due to general toxicity to the cells. At the same time, inhibition of NF-*κ*B activity by **1** and **3** seems to be specific (Table 1).

Most of the stilbenoids inhibited intracellular generation of reactive oxygen species (ROS) in PMA induced HL-60 cells. The strongest antioxidant effect was demonstrated by **2**, **16** and **17** which was significantly higher than the standard Trolox (Table 1). Several other stilbenoids (**1**, **3**, **4**, **6**–**10**, **21**–**24**) also demonstrated significantly high antioxidant properties which were comparable to Trolox. In the same assay, the majority of stilbenoids demonstrated moderate cytoxicity towards HL-60 cells but the antioxidant effect was observed at much lower concentrations confirming that antioxidant effect was not related to cytotoxic effect. Among the stilbenoids tested, **1**, **10**, **13**, **15**, **17** and **24**, were more cytotoxic than others towards HL-60 cells (Table 1). A remarkable structure-activity relationship was observed while testing two pairs of compounds, the stilbenoids **13** and **14**, and their bibenzyl analogues **17** and **16**, respectively. While compounds **17** and **16** demonstrated significantly higher antioxidant activity compared to Trolox, corresponding stilbenoids **13** and **14** were inactive (Table 1). The same compounds demonstrated reversed structure-activity relationship when tested for inhibition of iNOS activity as well as the inhibition of NF-*κ*B-dependent transcription assay and cytotoxicity to corresponding cell lines (Table 1). In these tests stilbenoids **13** and **14** were moderately active, while bibenzyls **17** and **16** were inactive. Supposedly, the lack of conjugation between the benzyl rings is responsible for the observed effects.

More than half of the peanut stilbenoids and most of other stilbenoids tested inhibited the activity of inducible nitric oxide synthase (iNOS) in LPS-induced macrophages resulting in a decrease of nitric oxide (NO) levels. Arachidin-1 (**1**) was the most potent inhibitor (IC_{50} = 1.9 μg/mL) in this test. Many other compounds also demonstrated considerable inhibition of iNOS activity (Table 1). Although cytotoxicity was also observed for some of the compounds but the concentration responsible for cytotoxicity was higher than the concentration responsible for inhibition of iNOS activity in the same assay which indicates that the inhibition of iNOS was not due to the cytotoxic effect. However, no cytotoxicity was observed for **2**, **3**, **15**–**17**, **21** and **24** (Table 1). The majority of stilbenoids did not

demonstrate appreciable selective anticancer properties in human solid tumor cell lines. The fact that they are not cytotoxic, and at the same time possess strong antioxidative properties and inhibition of iNOS activities that results into a decrease in the cellular nitrite level, allows to suggest their chemopreventive health benefits and warrants further tests (including *in vivo* effects).

Moderate cytotoxicity was observed among limited number of peanut stilbenoids and stilbene derivatives **11**–**24** (Figure 1) in a panel of mammalian kidney cells (Vero and LLC-PK_{11}) and cancer cells (SK-MEL, KB, BT-549, and SK-OV-3) up to the peak concentration tested (25 μg/mL). The highest, but moderate cytotoxicity was exhibited in all cell lines by arachidin-1 (**1**). At the same time its close 3′-dehydroxy analog **3** (Figure 1) was not cytotoxic (Table 1). Arachidin-2 (**2**) demonstrated comparable, but significantly lower cytotoxicity than that of **1** (Table 1). The only difference between moderately cytotoxic **2** and non-toxic **3** is the position of the double bond in the prenyl chain at C-4 in the stilbene skeleton (Figure 1). It seems that the interrupted conjugation in **2** provides cytotoxic properties to this molecule compared to **3**, which bears a longer conjugation system of alternating single and double bonds. In addition to arachidins 1–3 (**1**–**3**) only **5**, **10**, **11, 15**, **22**, and **24** were moderately toxic at higher concentrations (up to 25 μg/mL). Compounds **5**, **10**, **11**, and **22** were also moderately toxic to other cell lines tested (Table 1). The rest of stilbenoids did not demonstrate appreciable selective anticancer properties in human solid tumor cell lines.

In our experiments overall activity of **3** and **24** was about equal in cytotoxic, anti-inflamatory, and antioxidant assays, while **1** demonstrated overall higher activity (Table 1). Similar results (19) were obtained for these stilbenoids. All three stilbenoids showed concentration-dependent growth inhibitory effects on HL-60 cells. Arachidin-**1** (1) was about 4-fold more active compared to arachidin-3 (**3**) and resveratrol (**24**). However, in addition to growth inhibition, **1** was found to be cytotoxic (19). The above compounds were also investigated for their antioxidant activity and cytotoxicity (18) and for antioxidant and anti-inflamatory activities (16). Both research groups reported that all three stilbenoids did not exhibit significant cytotoxicity to RAW 264.7 cells. At the same time, these compounds were found to be potent antioxidants (16,18) and anti- inflamatory agents (16). It has been demonstrated (17) that **1** and **24** were effective inhibitors of PGE_2- or NO-mediated inflammation. The results of present research as well as findings of other research groups indicate the merits of systematic investigation of natural stilbenoids for their anticancer activities.

Evaluation of affinity of compounds tested to opioid receptors

All data were obtained from three experiments using three replicate plates per concentration; standard deviations in all experiments were equal to zero. No correlation between affinity to the opioid receptor subtypes and log P for all tested compounds was observed (Figure 3). Such behavior can be explained by the nature of the specific mechanisms of receptor activation with corresponding compounds.

The assay that was used in this study is an important pharmacological tool that measures the relationship of binding between the radiolabeled ligand and the test sample. If a compound has activities on sites remote from the actual binding site, then the ability of the radioligand to bind to the active site may be increased. As such, a control value for the radioligand may be close or equal to 100%, while a test compound may cause this value to increase to a higher than 100% level, which results in a negative value for that compound. Among all tested compounds, the most striking effect on κ opioid receptor systems was exhibited by *trans*-3,4′-dihydroxystilbene **15**;the effect is reflected in Figure 3 as a negative 697% value. This effect cannot be reasonably defined in terms of structure – activity association.

However, it is likely that the specific position and number of hydroxy groups in the structure of the simple stilbenoid **15** may be responsible for such a distinctive response. Stilbenoid **15** might have usefulness as an allosteric modulator, which could be employed to enhance the effects of known analgesic agents such as morphine. The low cytotoxicity of **15** (Table 1) allows the suggestion that this compound could be used in vivo. Combined use of **15** and analgesic agents may result in lower amounts of the latter needed to block pain, which is a benefit to the patient since it would reduce the potential for addiction. Compared with other compounds, **15** also demonstrated the highest degree (64.2%) of affinity to Δ-opioid receptors and moderate affinity (16.7%) to μ-receptors (Figure 3). Binding of the standard, naloxone, an extremely potent μ-opioid (and to a lesser degree κ- and Δ-opioid) receptor competitive antagonist, was close to 100% (Figure 3). Compounds **11**–**24** also showed some degree of activity as well (Figure 3). All of these compounds, with the exception of **21**, **23**, and **24**, demonstrated over 50% competitive binding to μ-receptors. In contrast, rhapontin (**23**) showed appreciable negative values for Δ- and μ-opioid receptors (Figure 3). Chlorophorin (**22**) had equally high affinity to both δ- and κ-opioid receptors, but no affinity to μ-receptors. Several of the other compounds demonstrated considerably high competitive antagonism at μ- and κ-sites (Figure 3). All of the peanut metabolites (Figure 1) were satisfactory Δ-opioid receptor competitive blockers (Figure 3) as well as agonists at μ- and κ-receptor subtypes. Two of the nine stilbenoids, **1** and **3**, exhibited high (−80.0 and −58.3%, respectively) non-competitive binding to μ-opioid receptor. Taking into account their low cytotoxicity and selective affinity to different types of receptors, some of the compounds tested may be of interest as prospective candidates for in vivo assays.

Evaluation of toxicity of tested compounds to mosquito larvae and adults

As an extension of our quest to explore the beneficial properties of peanut stilbene-derived compounds, we evaluated their toxicity to adult mosquito and mosquito larvae. While a lack of log P–affinity to opioid receptors in compounds tested was not surprising, some degree of lipophilicity–activity association in the mosquito larvae and adult mosquito assays was anticipated. Indeed, compounds with log P >3.14 (**13**, **14**, **16**, **18**, **19**, and **21**) showed the highest activity (Figure 4B-C) with the exception of chlorophorin (**22**), which was less active despite a high log P value of 3.29 (Figure 1). Compounds with lower lipophilicity, such as **11**, **24**, **23**, and **5** (Figure 1), demonstrated significantly lower toxicity (Figure 4A). There was a positive correlation between the lipophilicity of tested stilbene derivatives and their toxicity to mosquito larvae.

Although a structure–activity relationship cannot be suggested for the examined compounds, we note that stilbenoid **13** was significantly more active than its bibenzyl analogue **17**. At the same time, stilbenol **14** showed about equal toxicity to that of hydroxybibenzyl **16**. These two pairs of compounds provide a good model for estimation of the importance of the olefinic double bond for the toxicity of the stilbenoids in this assay. While compounds with virtually matching activities, **14** and **16** have close log P values (3.22 and 3.19, respectively), considerably more active stilbenoid **13** is characterized by a significantly higher lipophilicity (log P = 3.14) than its bibenzyl derivative **17** (log P = 3.07). Although the number of examined samples was limited, a suggestion can be made that the olefinic double bond in stilbenes is not essential for toxicity against mosquito larvae.

The activities of compounds **13**, **14**, **16**, and **18** are very low compared to the positive control permethrin. Stilbenoids **19**, **21**, and **22** display somewhat greater activity, but the effects were still relatively modest. The remaining compounds were inactive in these assays. At first glance, the lipophilicity data seems to play a role in the toxicity of natural peanut stilbenoids to adult mosquito as well (data not shown). Peanut stilbenoids with higher lipophilicity (log P values are given in parenthesis followed by mortality rate ± SD, %), including **5** (3.46; 6.7±5.8), **9** (2.59; 10±0.0), and **10** (3.34; 5.8±1.8), demonstrated slightly

higher toxicity than the less lipophilic **1** (2.44), **7** (1.36), and **8** (1.59), which were marginally active. However, similar results were obtained with acetone/DMSO control that gave 5.8±1.8% mortality, while untreated control was inactive and permethrin standard (6.25 μg/mosquito) caused 100% mortality. On the basis of these results, stilbenoids tested (Figure 1) do not seem to be insecticidal in the adult mosquito assay.

A systematic study of biological activity of new and known peanut stilbenoids was performed. This research revealed diverse biological activity of peanut stilbenoids as well as other natural and synthetic stilbene derivatives. Despite close structural similarities, individual stilbenoids demonstrated significantly different activities in the various assays employed. The results of the present research are consistent with published data on the bioactivity of some natural stilbenoids from peanut and other plant sources (1,8,10,18,19).

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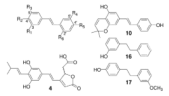

Compound	R_1	R_2	R_3	R_4	R_5	R_6	Log P (mean±SD)
1 (arachidin-1)*	OH		OH	OH	OH	H	2.44±0.001
2 (arachidin-2)*	OH		OH	H	OH	H	2.56±0.001
3 (arachidin-3)*	OH		OH	H	OH	H	2.83±0.000
4 (SB-1)*							2.59±0.001
5 (chiricanine-A)*	OH		OH	H	H	H	3.46±0.001
6 (arabypin-1)*	OH		OH	H	H	H	3.71±0.000
7 (arabypin-2)*	OH	H	OH		OH	H	1.36±0.002
8 (arabypin-3)*	OH		OH	H	OH	H	1.59±0.001
9 (arabypin-4)*	OH		OH	H	H	H	2.59±0.000
10 (arabypin-5)*							3.34±0.001
11 (pinosylvin)**	OH	H	OH	H	H	H	2.25±0.001
12 [(*E*)-3,4-stilbendiol]	OH	OH	H	H	H	H	Not determined
13 [(*E*)-5-methoxy-4′-stilbenol]	H	OH	H	OCH_3	H	H	3.14±0.001
14 [(*E*)-3-stilbenol]	OH	H	H	H	H	H	3.22±0.001

15 [(*E*)-3,4′-stilbendiol]**	OH	H	H	H	OH	H	2.08±0.001
16							3.19±0.001
17**							3.07±0.001
18 (3,5-*O*-dimethylpinosylvin)**	OCH_3	H	OCH_3	H	H	H	4.15±0.003
19 (3-*O*-methylpinosylvin)**	OH	H	OCH_3	H	H	H	3.21±0.000
20 [(*E*)-3,5,4′-stilbenetriol]	OCH_3	H	OCH_3	H	OCH_3	H	4.07±0.000
21 (pterostilbene)*	OCH_3	H	OCH_3	H	OH	H	3.15±0.001
22 (chlorophorin)**	OH		OH	H	OH	OH	3.29±0.003
23 (rhapontin)**	OH	H	O-Glc	OH	OCH_3	H	0.74±0.002
24 (resveratrol)*	OH	H	OH	H	OH	H	1.35±0.000

Figure 1.
Structures of tested compounds. *Peanut phytoalexins. **Other natural plant stilbenoids. O-Glc refers to a glucosyl moiety.

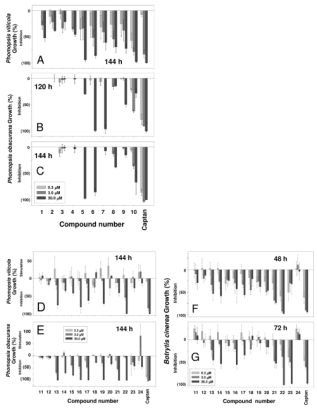

Figure 2.

Antifungal properties of peanut phytoalexinsagainst *Phomopsis viticola* (**A**) and *P. obscurans* (**B** and **C**). Antifungal properties of tested natural and synthetic stilbenoidsagainst *Phomopsis viticola* (**D**), *P. obscurans* (**E**), and *Botrytis cinerea* (**F** and **G**).

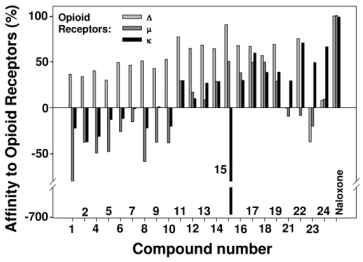

Figure 3.

Affinity of compounds tested to opioid receptors. Compounds **3** and **20** were not tested in this assay due to their insufficient quantity.

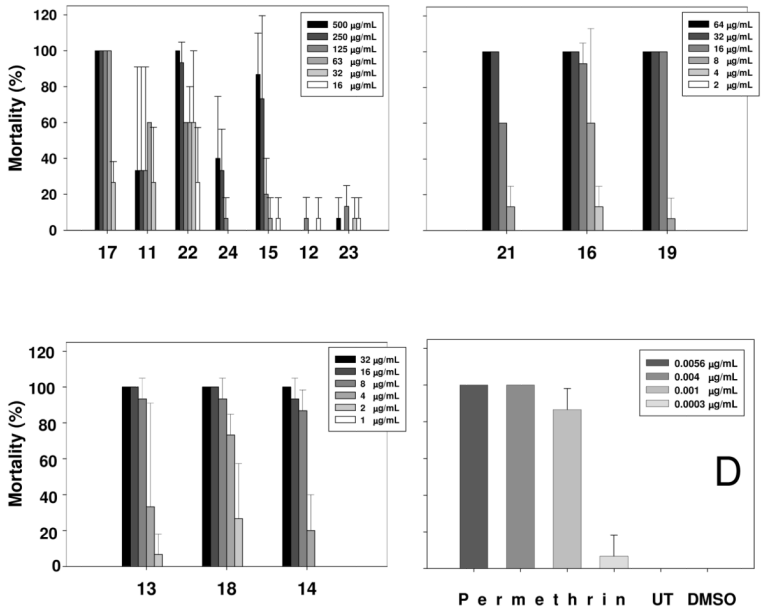

Figure 4.

Toxicity of compounds tested to mosquito larvae; panel **D** represents toxicity data for positive and negative standards. UT means Untreated; DMSO means dimethyl sulfoxide. Compound **20** was not tested in this assay due to its insufficient quantity.

Table 1

Cytotoxic, Anti-inflammatory, and Antioxidant Activities of Tested Compounds.

Cytotoxicity (anti cell proliferation activity) in a panel of cell lines							Inhibition of NF-κ B activity in SW1353 cells		Antioxidant Activity (inhibition of ROS generation) in HL-60 cells		Inhibition of iNOS activity (NO production) in RAW 264.7 cells	
	IC_{50} (μg/mL)						IC_{50} (μg/mL)		IC_{50} (μg/mL)		IC_{50} (μg/mL)	
Compound	**SK- MEL[1]**	**KB[1]**	**BT- 549[1]**	**SK- OV-3[1]**	**VERO[2]**	**LLC-PK11[2]**	**NF-κB**	**SP-1**	**Antioxidant activity**	**Cytotoxicity**	**iNOS**	**Cytotoxicity**
1 (arachidin-1)	15.0	4.8	4.8	14.5	12.0	2.8	12.0	-	0.45	5.2	1.9	8.3
2 (arachidin-2)	20.0	22.5	7.5	-	13.5	13.5	0.025	<0.025	0.04	16.0	4.1	-
3 (arachidin-3)	-	-	-	-	-	-	18.0	-	0.17	-	6.0	-
4 (SB-1)	-	-	-	-	-	-	-	-	0.3	-	>25	-
5 (chiricanine A)	20.0	-	-	-	-	19.5	-	-	-	19.0	6.5	13.0
6 (arahypin-1)	>25	-	-	-	-	-	-	-	0.35	>31.3	-	-
7 (arahypin-2)	>25	-	-	-	-	-	-	-	0.19	-	-	-
8 (arahypin-3)	-	-	-	-	-	-	-	-	0.4	-	-	-
9 (arahypin-4)	-	-	-	-	-	-	-	>25	9.5	>31.3	-	-
10 (arahypin-5)	17.5	>25	>25	-	>25	19.5	-	-	1.3	4.5	7.0	16.0
11	12.5	13.8	13.0	17.0	13.0	10.5	14.5	12.5	-	18.0	6.9	10.5
12	-	-	-	-	-	-	-	-	20.0	>25	-	-
13	-	-	-	-	-	-	19.5	25.0	-	4.0	8.0	19.5
14	-	-	-	-	-	-	>25	>25	-	19.0	11.4	22.0
15	-	-	-	-	19.5	12.0	-	-	-	4.0	10.3	-
16	-	-	-	-	-	>25	-	-	0.012	17.0	19.5	-
17	-	-	-	-	-	-	-	-	0.027	9.0	20.0	-
18	-	-	-	-	-	-	-	-	-	>25	>25	-
19	-	-	-	-	-	-	17.0	23.0	-	18.0	8.5	12.0
21 (pterostilbene)	-	-	-	-	-	-	-	-	0.3	21.0	9.9	-
22	-	>25	20.0	>25	15.5	14.5	22.5	-	0.8	25.0	4.8	9.0
23	-	-	-	-	-	-	-	-	0.55	20.0	-	-
24 (resveratrol)	-	-	-	-	-	>25	18.5	>25	0.28	8.0	10.0	-

Cytotoxicity (anti cell proliferation activity) in a panel of cell lines							Inhibition of NF-κ B activity in SW1353 cells		Antioxidant Activity (inhibition of ROS generation) in HL-60 cells		Inhibition of iNOS activity (NO production) in RAW 264.7 cells	
	IC_{50} (μg/mL)						IC_{50} (μg/mL)		IC_{50} (μg/mL)		IC_{50} (μg/mL)	
Compound	**SK- MEL[1]**	**KB[1]**	**BT- 549[1]**	**SK- OV-3[1]**	**VERO[2]**	**LLC-PK11[2]**	**NF-κB**	**SP-1**	**Antioxidant activity**	**Cytotoxicity**	**iNOS**	**Cytotoxicity**
Doxorubicin[3]	0.8	1.45	1.35	1.3	>5	0.9				0.24		
Parthenolide[3]							0.8	8.35			0.42	13.5
Trolox[3]									0.11			

[1] Cancer cells.

[2] Noncancer cells. SK-MEL – Human malignant melanoma; KB – Human oral epidermal carcinoma; BT-549 – Human breast ductal carcinoma; SK-OV-3 – Human ovary carcinoma; Vero – Monkey kidney fibroblasts; LLC-PK11 – Pig kidney epithelial cells.

[3] Standard compounds with known biological activities. Hyphen (-) means no activity up to 25 μg/mL.