PROCEEDINGS OF THE NEUROSCIENCE SOCIETY OF NIGERIA (NSN) CONFERENCE, JOS, 2006

Bioactivity Screening of Some Medicinal Plants using The Brine Shrimp Toxicityand Antioxidant Tests

By

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Abstract

Plants have undoubtedly become reliable sources of new bioactive compounds or even known compounds with new activities. In furthering the search for such compounds, some plants, known from ethnobotanical studies to be useful in the treatment of various diseases and ailments, were screened for bioactivity using the brine shrimp toxicity test and the antioxidant (radical scavenging) test. Altogether 26 plants were used 17 were screened for brine shrimp toxicity while 16 were screened for antioxidant activity. The 70% ethanol extract of each plant was used. In addition standard tests for the presence of secondary metabolites were carried out. The plants were all found to demonstrate varying levels of activity in the test systems. In the brine shrimp lethality test *Citrus sinensis* was most active (LC_{50}=16.8 µg/mL) while *Vernonia amygdalina* was the most potent in the sedative estimate (LC_{50}=3.0 µg/mL). In the antioxidant screening *Zingiber officinale* showed the greatest potency. While brine shrimp lethality could be used as a general indication of biological activity, plants with significant sedative effect on brine shrimp are potentially CNS-active. Antioxidant (radical scavenging) activity has been linked to inflammation and neuro-degenerative disorders.

Introduction

The process of obtaining a pure, pharmacologically active compound from a medicinal plant is often long and tedious. The classical approach has often been to carry out purely phytochemical studies and then subject isolated compounds to biological testing. However this approach tends to yield constituents that can be obtained fairly readily and in relatively high quantities. Unfortunately such compounds are often not the active compounds in medicinal plants (Christophersen *et al.*, 1991). Therefore researchers now favour the bioassay-guided approach. This approach requires that the crude plant extracts as well as all fractions, sub-fractions and pure compounds have to be tested, and in that sequence. This means that only when the extract has been shown to be active can it be fractionated. Also only active fractions should be further fractionated until pure compounds are obtained. Thus a bioassay-guided isolation requires, first and foremost, that a reliable, functional bioassay be available (Hamburger and Hostettmann, 1991).

Bioassays can be defined as those tests which are used to detect biological activity of an extract or pure substance isolated from an extract, obtained from a living organism. Although clinical trials and *ex vivo* tests which utilize isolated tissues/organs can be brought into this definition, they are usually not included. Among other things the assay must be simple, rapid, reproducible and relatively inexpensive. A number of assays have been found to fulfill these requirements. They are collectively known as 'benchtop' bioassays. The brine shrimp lethality test is arguably the most widely used among them. Others include: Crown gall tumour test; Frond inhibition of *Lemna* (duckweed) test; and Yellow fever mosquito larvicidal test (McLaughlin *et al.*, 1998).

Since bioactive compounds are almost

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always toxic in high doses, *in vivo* lethality in a simple organism such as the brine shrimp can be used as a convenient means of monitoring bioactive constituents of plants (Mclaughlin *et al.*, 1998). Eggs of the brine shrimp, *Artemia salina* Leach, are viable for several years when kept in a dry state. When placed in sea water they hatch within 48 hours to provide a large number of larvae (nauplii) which remain very active for at least another 24 hours. Brine shrimp lethality test was first used by Michael *et al.*, (1956) and later developed by Vanhaecke *et al.*, (1981) and Sleet and Brendel (1983). It has been used for the detection of fungal toxins (Harwig and Scott, 1971), cyanobacteria toxins (Jaki *et al.*, 1999), cytotoxicity (McLaughlin *et al.*, 1991; Pelka *et al.*, 2000), heavy metals (Martinez et al., 1998), and pesticides (Barahona and Sanchez-Fortune 1999). Substances that slow down the activity of nauplii are suspected to have Central Nervous System activity. The brine shrimp lethality test has advantages of being rapid (takes only 24 hours), inexpensive (eggs and other materials used are cheap), simple (a soap dish is used and no aseptic techniques are required), and only a small amount of sample (2-20 mg) is needed. In addition animal rights advocates have not yet objected to the use of invertebrates in experimental work.

The test for antioxidant activity uses the stable radical 2,2'-diphenyl-1-picrylhydrazyl (DPPH) as test reagent. This substance helps to determine the radical scavenging potential of the test substance. The test is simple and easily carried out on thin-layer chromatograms. The DPPH reagent forms complexes with the antioxidant compounds and this gives rise to colour formation. Antioxidants are radical scavengers that protect the human body against free radicals that cause pathological conditions such as ischaemia, inflammation, neuro-degeneration, Parkinson's disease, the aging process and perhaps dementias (Polterat, 1997). Plant secondary metabolites, notably phenolics such as flavonoids have radical scavenging (antioxidant) properties (Nakayoma and Yamada, 1995; Oke and Hamburger, 2002).

Plants selected for the screening are known, from ethnobotanical studies, to be useful in the treatment of various diseases and ailments. Some have also been shown to possess certain biological activities. However, the advantage of screening these plants, using the chosen test systems is that new bioactive compounds can be obtained which could find use in other ways hitherto unknown.

Materials and Methods

Plant material

The plants used were collected around Jos, Nigeria. Authentication was done by comparison with herbarium specimens deposited in the herbaria of the Department of Pharmacognosy, University of Jos and the Federal College of Forestry, Jos. Altogether twenty-six (26) plants were selected for the screening tests, seventeen (17) for brine shrimp test and sixteen (16) for antioxidant test. The plants selected for screening are *Anogeissus leiocarpus, Azadirachta indica, Boswellia dalzielii, Canarium schweinfurthii, Carica papaya, Citrus sinensis, Datura innoxia, Datura stramonium, Dichrostachys cinerea, Eucalyptus globulus, Ficus thonningii, Garcinia kola, Jacaranda mimosifolia, Lantana camara, Mangifera indica, Manihot esculenta, Ocimum gratissimum, Parinari curatellaefolia, Persea americana, Psidium guajava, Ricinus communis, Securidaca longipedunculata, Steganotaenia araliacea, Vernonia amygdalina, Vitex doniana and Zingiber officinale.* The required plant parts (leaves, bark or fruit) were air-dried, chopped to smaller particles or ground to powder. Extraction was carried out by maceration using 70% ethanol; thereafter the extract was concentrated to dryness *in vacuo* on a rotary evaporator. The dry extracts were stored in a desiccator until when required for further use.

Phytochemical screening

The powdered plant materials and extracts were

screened for the presence of the following secondary metabolites, employing the appropriate tests as indicated below:
Alkaloids: Dragendorff's, Wagner's and Meyer's tests
Anthraquinones: Borntrager's test
Glycosides: Liebermann-Burchard/ Fehling's test; Keller-Killiani test; Salkowski's test
Flavonoids: Shinoda's test
Saponins: Froth test
Tannins: Ferric chloride test

All tests were carried out as described by Sofowora (1993) and Evans (2002).

Brine shrimp test
Brine shrimp (*Artemia salina* Leach) eggs, bought from a commercial pet shopin the USA, were placed in saline water (1% $^{w}/_{v}$ NaCl) in a soap dish. After 48h incubation at room temperature the hatched larvae (nauplii) were attracted to one side of the vessel by a light source and collected with a pipette. Ten nauplii were transferred with some seawater, *ca.*2mL, into each test tube. Specific volumes of sample solution, prepared in sea water, were added to the nauplii such that final concentrations of 10, 100 and 1000 g/mL in a total volume of 5mL per test tube would be obtained. Each treatment was carried out in duplicate with saline water as control. After incubating for 24 h the number of dead nauplii in each test tube was counted and recorded for the lethality test. Also the number of nauplii with sluggish motion was counted for the sedative estimate. The data was processed using Finney Probit Analysis computer programme to calculate median lethal concentration (LC_{50}) values with 95% confidence intervals for statistically significant comparisons of potency (Meyer *et al.*, 1982; McLaughlin *et al.*, 1991; Wanyoike, *et al.*, 2004).

Antioxidant test
The test for antioxidant/ radical scavenging properties of extracts, fractions and isolated compounds were carried out on thin-layer chromatography (TLC) plates using two spray reagents: -carotene (0.1% $^{w}/_{v}$ in MeOH or EtOH) and 2,2'-diphenyl-1-picrylhydrazyl (DPPH) (Burits and Bucar, 2000). Each of the two spray reagents was used independently. The samples to be tested were spotted on a TLC plate. Ascorbic acid is also spotted along as a positive control. A duplicate plate was prepared for the second spray reagent. The plates were then developed using a suitable solvent system as in normal TLC. After development, the plates were air-dried at room temperature. One plate was sprayed with DPPH while the other is sprayed with -carotene. For the plate sprayed with -carotene, antioxidant spots appear bright yellow against a pale yellow background. By irradiating the plate with uv light at 366nm for 15min, the pale yellow background was bleached and the antioxidant spots become clearly visible. For the plate sprayed with DPPH, antioxidant spots showed up as yellow against a purple background. Since the time taken for the yellow colour to develop varies among different compounds depending on their antioxidant power, the appearance of the yellow colour can be timed. The time taken relative to ascorbic acid may be interpreted as an index of antioxidant capability. In addition, for samples that gave positive test on TLC, the antioxidant capability was further assessed by preparing a 1% $^{w}/_{v}$ solution in ethanol or methanol. DPPH reagent was added and the absorbance measured in a colorimeter at a wavelength of 517nm. The absorbance was taken again after 20min. The percentage decrease in absorbance was then calculated using ascorbic acid as positive control and methanol or ethanol as blank (Oke and Hamburger, 2002; Li *et al.*, 2004).

Results
The results of phytochemical screening for various secondary metabolites are given in Table 1. The plants were all tested for the presence of alkaloids, anthraquinones, glycosides, flavonoids, saponins and tannins. The results of brine shrimp (cytotoxicity) test

are given in Table 2. Both the lethality and sedative estimates, expressed as LC_{50}, were determined for the plants. The results of antioxidant screening for the plants being investigated are given in Table 3. Antioxidant ratings are recorded as strong, medium or weak, or negative where the plant is devoid of antioxidant activity.

Table 1: Phytochemical screening of plants for secondary metabolites

Plants	Alkaloid	Anthraquinone	Glycoside	Flavonoid	Saponin	Tannin
Anogeissus leiocarpus	++	-	+	-	++	++
Azadirachta indica	++	-	++	++	++	+++
Boswellia dalzielii	-	-	++	-	++	+++
Canarium schweinfurthii	-	-	+	++	++	+
Carica papaya	++		++	++	++	++
Citrus sinensis	-	-	-	++	-	++
Datura innoxia	++		++	++		++
Datura stramonium	++	-	++	++	-	++
Dichrostachys cinerea	-	-	++	++	++	++
Eucalyptus globulus	-	-	+++	++	+++	++
Ficus thonningii	-	-	++	+		+
Garcinia kola	++	++	-	++	-	+++
Jacaranda mimosifolia	-	-	+++	-	-	++
Lantana camara	+	+	-	-	+++	+++
Mangifera indica	-	-	++	+++	++	+++
Manihot esculenta	-	-	+++	-	-	++
Ocimum grastissimum	-	-	+	++	+	+++
Parinari curatellaefolia	-	-	++	-	++	++
Persea Americana	-	-	++	++	++	++
Psidium guajava			+++	+++	+++	+++
Ricinus communis	++	-	++	++	-	++
Securidaca longipedunculata	-	-	+	-	++	+
Steganotaenia araliacea	-	-	++	-	+++	++
Vernonia amygdalina	-	-	++	-	+++	+++
Vitex doniana	++	++	++	+	++	++
Zingiber offininale	+++	+	-	++	-	++

+++ = present (very significant); ++ = present (moderaely significant); + = present (weakly significant); - = absent

Table 2: Brine shrimp cytotoxicity test of plants lethality and sedative estimates

Plant	Plant part used	Family	Brine Shrimp test LC_{50} (g/mL)	
			Lethality	Sedative estimate
Azadirachta indica	Leaves	Meliaceae	183.3	48.8
Boswellia dalzielii	Bark	Burseraceae	429.6	214.8
Carica papaya	Leaves	Caricaceae	81.6	36.7
Citrus sinensis	Leaves	Rutaceae	16.8	16.8
Datura stramonium	Leaves	Solanaceae	63.1	63.1
Eucalyptus globulus	Leaves	Myrtaceae	72.9	5.0
Garcinia kola	Seeds	Sterculaceae	96.0	96.0
Lantana camara	Leaves	Verbenaceae	29.6	5.0
Mangifera indica	Leaves	Anacardiaceae	117.5	26.1
Manihot esculenta	Leaves	Euphorbiaceae	74.7	77.8
Ocimum grastissimum	Leaves	Labiatae	63.1	5.0
Psidium guajava	Leaves	Myrtaceae	96.0	74.5
Ricinus communis	Leaves	Euphorbiaceae	77.8	21.0
Steganotaenia araliacea	Bark	Umbeliferae	37.2	10.3
Vernonia amygdalina	Leaves	Compositae	96.7	3.0
Vitex doniana	Leaves	Verbenaceae	104.9	41.4
Zingiber offininale	Rhizome	Zingiberaceae	63.1	5.0

* Value computed by Finney computer programme for Probit Analysis

Table 3: Test for antioxidant activity of selected plants

Plant	Plant part used	Family	Antioxidant test
Anogeissus leiocarpus	Leaves	Combretaceae	-
Boswellia dalzielii	Bark	Burseraceae	++
Canarium schweinfurthii	Leaves	Burseraceae	-
Carica papaya	Leaves	Caricaceae	+
Datura innoxia	Leaves	Solanaceae	-
Dichrostachys cinerea	Leaves	Leguminosae	++
Ficus thonningii	Leaves	Moraceae	-
Jacaranda mimosifolia	Leaves	Bignoniaceae	-
Parinari curatellaefolia	Leaves	Rosaceae	-
Persea americana	Leaves	Lauraceae	++
Psidium guajava	Leaves	Myrtaceae	+++
Ricinus communis	Leaves	Euphorbiaceae	++
Securidaca longipedunculata	Bark	Polygalaceae	-
Steganotaenia araliacea	Bark	Umbeliferae	++
Vernonia amygdalina	Leaves	Compositae	+
Zingiber offininale	Rhizome	Zingiberaceae	+++

+++ = strongly antioxidant; ++ = moderately antioxidant; + = weakly antioxidant
- = not antioxidant

Discussion

The results of Phytochemical screening (Table 1) show that all the plants contain tannins, majority contain saponins, flavonoids and glycosides while a few have alkaloids and anthraquinones. Plant secondary metabolites from higher plants have played, and continue to play important role yielding novel chemical structures that serve as templates for molecular modification to give better drugs. For instance, records have revealed that six of the top twenty drugs sold by pharmaceutical companies in 1996 were natural products. In addition more than 50% of these top twenty drugs were linked directly to natural products research (Phillipson, 1999). Thus the selected plants represent a potential source of compounds capable of serving as drugs.

From Table 2, the brine shrimp lethality test indicates that *Citrus sinensis* has the lowest LC_{50} value of 16.8µg/mL. This implies that it is the most potent in this assay. Thus it might be said that this plant and others with similar, low LC_{50} values (such as *Lantana camara* LC_{50} 29.6µg/mL) could provide newer bioactive compounds. In the sedative estimate, *Vernonia amygdalina* was highly potent with an LC_{50} of 3.0µg/mL, followed by *Eucalyptus globulus*, *Lantana camara*, *Ocimum gratissimum* and *Zingiber officinale* LC_{50} 5.0µg/mL. The brine shrimp test is primarily an indication of bioactivity; however it has been observed that plants and compounds demonstrating potent brine shrimp activity have good correlation with anticancer activity. Also compounds that sedate brine shrimp nauplii are likely to have activity on the central nervous system (McLaughlin *et al.*, 1991). This shows that the plant, *V. amygdalina*, probably contains compounds which can act on the Central Nervous System since it is very potent in the sedative estimate (see Table 3). Other good candidates are *E. globulus*, *L. camara*, *O. gratissimum* and *Z. Officinale*.

In the antioxidant assay, *Zingiber oficinale* was found to be most potent, followed by *Psidium guajava*. Many phenolic compounds are known to be potent antioxidants (Nakayoma and Yamada, 1995; Oke and Hamburger, 2002). This is also borne out by the results of this study because the strongly antioxidant compounds are also rich in tannins and flavonoids, which are phenol-derived constituents. Antioxidant compounds are believed to play significant roles in preventing/alleviating several diseases including liver problems, brain dysfunction, cancer, artherosclerosis, rheumatoid arthritis, diabetes mellitus and AIDS (Burits and Bucar, 2000). Thus many of the plants studied could be potential sources of antioxidants needed to combat diseases affecting humans.

In conclusion therefore, the results of this study highlight the great potential of these plants as sources of new bioactive compounds or known compounds with new applications, especially in diseases which affect the central nervous system.

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