

## Biological activities and phytochemical constituents of the gray mangrove *Avicennia marina* (Forssk.) Vierh.

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### ABSTRACT

*In vitro* assessment of the antibacteriophage, antibacterial and anticandidal activities as well as cytotoxicity were evaluated for both aqueous and ethanol extracts prepared from roots, cotyledons, leaves and stems of *Avicennia marina*. Aqueous extracts of both shoots and roots of the seedlings demonstrated antibacteriophage activity using coliphage against *Escherichia coli* NRRL B-3704, which indicates antiviral activity. Aqueous extracts also exhibited moderate cytotoxicity against the larvae of the brine shrimp *Artemia salina*, which demonstrates antiplasmodial and antimalarial activities. However, both aqueous and ethanol extracts of various parts of the seedlings lacked antimicrobial activities against eight microbial test strains. On the other hand, irradiation of either seedling parts or plant extracts with long-wave UV radiation elicits immediate phototoxic activity. Investigating the production of growth-regulating substances in crude extracts reveals the presence of auxin- and cytokinin-like activities. Phytochemical analysis of ethanol extract of the shoot system indicates the presence of some biologically active metabolites including tannins, flavonoids, sterols, iridoid glycosides and organic acids. Alkaloids and saponins were not present in the extracts, which means no toxicity. The potential medicinal value and phytochemical prospects of the gray mangrove grown on the Red Sea coastline of Egypt are discussed.

**KEYWORDS:** antibacteriophage activity, phototoxic antimicrobials, cytotoxicity, growth regulators, Gulf of Aqaba, Red Sea.

### INTRODUCTION

Mangrove forests have been utilized for many functions including wood production, firewood and charcoal (Tomlinson 1994). However, wood-related activities or industries based on lingo-cellulose as a manufacturing substrate are very destructive and the rates of mangrove renewal do not match this at all (Kairo *et al.* 2001). Recently, it has been strongly recommended that mangroves should be considered as a valuable source for chemical constituents with potential medicinal and agricultural values (Miles *et al.* 1998). Although the chemical constituents of most mangrove plants still have not been studied extensively, investigations have led so far to the discovery of several novel compounds with prospective medicinal value for the discovery of new chemotherapeutic agents.

*Avicennia marina* (Forssk.) Vierh. (Avicenniaceae) has received some attention in determining its important chemical constituents. A naphthofuran compound with phytoalexin activity has been isolated (Sutton *et al.* 1985; Miles *et al.* 1998) and fatty acids, sterols and hydrocarbons studied in relation to their chemotaxonomic significance in eleven mangrove species including *A. marina* (Hogg & Gillan 1984). The presence or absence of an iridoid glucoside 2'-cinnamoyl mussaenosidic acid from *A. marina* extracts can be used in subspecific chemotaxonomy (Bousquet-Mélou & Fauvel 1998).

*In vitro* antimalarial activity and cytotoxicity of *A. marina* have also been reported and flavonoid glycosides having preliminary anticancer activity isolated (Sharaf *et al.* 2000). Recently, chemo-preventive activity (anti-tumor promoters) of some naphthoquinones and their analogs isolated from *Avicennia* plants was noted (Itoigawa *et al.* 2001). The bark and roots of *A. marina* are known to contain the tannin lapachol

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(Tomlinson 1994). The bark, leaves and fruits of *A. marina* are used in folk medicine to treat skin diseases.

The current study was planned to have more insight into the active metabolites of the gray mangrove *A. marina*. Application of a range of bioassays was intended to capture unexplored medicinal uses. The study aims to: a) assess antibiotic action and growth-regulating potential of both aqueous and ethanol extracts prepared from roots, cotyledons, leaves and stems; b) analyze its phytochemical constituents; and c) highlight potential uses and valuable chemical constituents of such endangered marine plant.

## MATERIALS AND METHODS

Seeds of *A. marina* were collected from El-Nabq Gulf of Aqaba, Red Sea in November 2002. Voucher specimens of the mature plant were deposited in the Botany Department Herbarium, Suez Canal University, Ismailia, Egypt. Seeds were transferred in moist plastic bags to the laboratory in Ismailia. One-year-old plants growing in the laboratory in plastic pots watered with 21 ‰ saline water were used as the experimental plant materials for the study. Plants of *A. marina* were maintained in a 16 h photoperiod at light radiation  $100 - 150 \mu\text{mol m}^{-2}\text{s}^{-1}$  and temperature  $30 \pm 5^\circ\text{C}$  during the day and  $20 \pm 5^\circ\text{C}$  at night.

Various parts of *A. marina* seedlings were extracted by maceration with ethanol (10 ml/g fresh weight) for 5 min in a homogenizer, left to stand overnight in the dark, then filtered (Harborne 1984). The filtrates were evaporated in a rotary evaporator under reduced pressure at  $40^\circ\text{C}$ . The residue was dissolved in 3 ml ethanol yielding the crude ethanol extract for further assays. Aqueous extracts of both shoot and root systems were also prepared. Both aqueous and ethanol crude extracts were tested immediately for various biological activities. Ethanol extracts of the shoot system (Figure 1 A) was used for the phytochemical analysis.

Table 1 lists the details of test organisms and specific bioassay methods performed in order to evaluate the biologically active metabolites that may be present in the tissues of *A. marina*.

The antibacteriophage bioassay was performed using coliphage against *Escherichia coli* NRRL B-3704 in the direct plaque assay (Ackermann & DuBow 1987), which indicates the antiviral activity of the mangrove extracts. The antibacteriophage assay may suggest possible models for anti-tumour action (Robertson *et al.* 1982). A combination of 0.5 ml of the host bacterial culture in the exponential growth phase and 0.1 ml of the phage in addition to 0.1 aqueous test extract were mixed thoroughly in a tube of 5 ml Lauria soft Agar (0.7 ‰). Then, the soft-agar medium was poured on a Lauria agar plate and left to solidify. The plates were incubated inverted at  $37.5^\circ\text{C}$  for 12-16 h. Clear areas of lytic plaques (pfu/ml) were counted in comparison with the control, which had 0.1 ml distilled water instead of the plant extract.

Cytotoxicity was assessed using the multi-well cytotoxic assay (Solis *et al.* 1993) carried out against the larvae of the brine shrimp *Artemia salina* (obtained from Interpet Ltd. Dorking, England). The bioassay demonstrates antimalarial activity and predicts antitumor and pesticidal potential. Brine shrimp eggs were hatched in seawater supplemented with 6 mg/l dried yeast, oxygenated with an aquarium pump and incubated in a warm room at  $22-29^\circ\text{C}$  for 48 hr. Serial dilutions in 100  $\mu\text{l}$  seawater were made into the 96 wells of microplates. Control wells with either seawater or Tween 80 were included in each experiment. About 100  $\mu\text{l}$  of the nauplii (containing 10-15 organisms) was added to each well and the multi-well plate incubated at  $22-29^\circ\text{C}$  for 24 hr. Plates were then examined under a binocular microscope and the numbers of dead (non-motile) nauplii in each well counted. Finally, all shrimps were scarified by adding 100  $\mu\text{l}$  methanol to each

well. After 15 min, the total numbers of shrimps/well were counted. Probit analysis (Finney 1971) was used for calculating the LC<sub>50</sub> values.

Antimicrobial activities of both aqueous and ethanol extracts of various parts of *Avicennia marina* plant were tested by the disc-diffusion method (Ericsson & Sherris 1971) against eight test strains. The antibacterial activity was assessed against five bacteria capable of human pathogenicity and one airborne bacterium, *Bacillus subtilis* (Table 3). Two yeasts were used for investigating the anti-candidal activity of the crude extracts. The microorganisms were obtained from the culture collection of the United States Department of Agriculture, Northern Regional Research laboratory (Peoria, Illinois, USA).

Table 1: Bioassays and test organisms used for *in vitro* assessment of various biological activities of *Avicennia marina* plant extracts

| Biological activity           | Bioassay  | Test organism  | Plant extract       | Biological Indication  |
|-------------------------------|---|--|---------------------|--|
| Antibacterial                 | Disc Diffusion/<br>Hole-plate/ (MIC)                              | Gram-positive & Gram-negative test bacterial strains             | Aqueous/<br>Ethanol | Bacteriostatic & Bactericidal (i.e. activity against prokaryotic cells)  |
| Anticandidal                  | Disc diffusion/<br>Hole-plate                                     | <i>Candida albicans</i> & <i>Candida tropicalis</i>              | Aqueous/<br>Ethanol | Anticandidal (i.e. activity against unicellular eukaryotic cells)  |
| Anti-bacteriophage            | Plaque assay  | Coliphage against <i>E. coli</i>                                 | Aqueous             | Activity against coliphages (i.e. antiviral activity)  |
| Cytotoxicity                  | Multi-well cytotoxicity assay                                     | nauplii of <i>Artemia salina</i>                                 | Aqueous             | Antiplasmodial, antifilarial & antimalarial activity   |
| <i>In vitro</i> Phototoxicity | Elicitation with long wavelength UV                               | <i>Candida albicans</i> & Gram-positive & Gram-negative bacteria | Ethanol             | Immediate induction of defence compounds (phytoalexins) by abiotic elicitor  |
| Plant growth regulators       | <i>In vitro</i> bioassay for induction of callus, roots & shoots. | Carrot hypocotyls  | Aqueous             | 1. Induction of roots (i.e. presence of auxin),<br>2. induction of shoots (i.e. presence of cytokinin),<br>3. growth inhibition (i.e. presence of inhibitors). |

An *in vitro* phototoxicity bioassay was applied in order to assess the influence of abiotic factors such as long-wave UV radiation on the induction of immediate antimicrobials. The *Candida* phototoxic assay adjusted by Knudsen (1985) was applied. The method depends mainly on measuring the area of the yeast/bacterial free zone. Crude ethanol extracts prepared from *A. marina* shoots were used to test phototoxicity. Filter paper discs loaded with 95 % ethanol were used as control. Six experimental replicate plates and six control plates were used for each treatment. Plates were irradiated for 1 min with UV radiation emitted from either a UV lamp (long wave source) or a solar simulator source. Cultures were incubated at 30°C for 18-48 hr in the dark. The area (cm<sup>2</sup>) of clear zone of inhibition surrounding the test material was determined for each treatment. The irradiated intensity was determined as energy fluency rate (i.e. energy density per unit time) with an international light model IL 1400A radiometer. It was 1.25 mW/min/cm<sup>2</sup> as determined from the UV lamp and 50 mW/min/cm<sup>2</sup> produced from the solar simulator. A detailed experimental study was undertaken recently in order to calibrate the minimal time of exposure and the optimal irradiating energy required for inducing the maximum inhibition of microbial growth (Khafagi et al. 2001).

Investigating the growth regulating potential of ethanol extracts of *A. marina* was done using *in vitro* growth assay of carrot explants in a growth regulator-free medium

containing the mangrove extract (Brain *et al.* 1973; Khafagi *et al.* 1999). Carrot hypocotyls were grown aseptically on tissue-culture media (Murashige & Skoog 1962) supplemented with 0.75 % agar and 3 % sucrose at pH 5.8. Extracts of various *A. marina* organs were tested for having growth-regulating substances. Induction of callus, roots, shoots or growth inhibition were tested in plates inoculated with 1-cm-long hypocotyls and supplemented with 1 ml aqueous mangrove extracts.

Phytochemical tests for alkaloids, flavonoids, saponins, tannins, sterols & terpenes and glycosides were carried out as described by Trease & Evans (1987) and Evans (1996). Each test was qualitatively expressed as negative (-) or positive (+). Subsequent thin-layer chromatographic (TLC) analysis was performed on silica-gel G pre-coated plates using the recommended TLC systems for major classes of plant chemicals described by Harborne (1984).

## RESULTS

The aqueous extracts of both shoots and roots have moderate antibacteriophage and cytotoxic activities. Aqueous extracts of shoots displayed more activity than that of the roots. *A. marina* shoots decreased the infectivity of phages (pfu/ ml) against their host bacteria more than root extracts (Table 2). The LC<sub>50</sub> of shoot extracts was more active than that of the roots (Table 2).

Table 2: Antibacteriophage and cytotoxic activity of *Avicennia marina* plant extracts

| Plant extract | Antibacteriophage activity                            |   | Cytotoxicity<br><i>Artemia salina</i> nauplii<br>LC <sub>50</sub> (µl/ml) |
|---------------|---|---|---|
|               | Coliphage against <i>Escherichia coli</i> NRRL B-3704 |   |   |
|               | Mean value for Control<br>(pfu/ml)                    | Mean value for 0.1 µl Plant<br>extract (pfu/ml) |   |
| Shoot system  | 850 ± 0.33  | 120 ± 0.27                                      | 12.50 ± 0.24  |
| Root system   | 850 ± 0.33  | 147 ± 0.16                                      | 25.25 ± 0.49  |

LC<sub>50</sub> is estimated from the brine shrimp micro-well assay by calculations using probit test.

Both aqueous and ethanol extracts of various plant parts lack antimicrobial activities against seven microbial test strains (Table 3). Alternatively, treatments of shoot and root extracts with long-wavelength UV radiation induced phototoxic activity, which may reflect immediate production of phytoalexin defence compounds (Table 3 & Figure 1).

Table 3: Antimicrobial activity and UV-dependent phototoxicity of *Avicennia marina* plant extracts. - = inactive. Numbers are the calculated area (cm<sup>2</sup>) of growth inhibition induced on irradiation of plant extracts in the presence of microorganisms.

| Test organism                | Source      | Antimicrobial Activity |                 | UV-dependent activity of shoot<br>extracts (cm <sup>2</sup> ) |  |
|------------------------------|-------------|------------------------|-----------------|---|--|
|                              |             | Shoot<br>extract       | Root<br>Extract | UVA lamp<br>(1.25 mW/ min/<br>cm <sup>2</sup> )               | Solar simulator<br>(50 mW/ min/<br>cm <sup>2</sup> ) |
| <i>Staphylococcus aureus</i> | NRRL B-767  | -                      | -               | 2.55  | 25.35  |
| <i>Bacillus subtilis</i>     | NRS- 744    | -                      | -               | 2.48  | 24.11  |
| <i>Escherichia coli</i>      | NRRL B-3704 | -                      | -               | 1.99  | 12.88  |
| <i>Klebsiella pneumonia</i>  | NRRL B-3521 | -                      | -               | 2.1   | 14.66  |
| <i>Proteus vulgaris</i>      | NRRL B-123  | -                      | -               | 1.89  | 10.23  |
| <i>Candida albicans</i>      | NRRL Y-477  | -                      | -               | 3.12  | 26.78  |

The carrot-hypocotyl bioassay shows that ethanol extracts of seeds and cotyledons have auxin-like and cytokinin-like activities and only scarce amounts of growth inhibitors. *In vitro* assays inoculated with 1-cm carrot hypocotyls and assayed for the growth-regulating activity of *A. marina* cotyledon extracts showed the production of some short

roots and many long and hairy roots (Figure 1 B1), comparable with exogenous application of 1 mg/l IAA or NAA. Induction of well-developed shoots (two-shoots/explant) was noticed from mixing *A. marina* extracts of both shoots and roots, and those producing shoots also induced some root formation too (Figure 1 B2). Root extracts alone induced dark callus formation in carrot hypocotyls (Figure 1 B3).

Phytochemical analysis of *A. marina* plant extracts reveals the presence of tannins, sterols, flavones, iridoid glycosides and organic acids, but volatile oils, alkaloids and saponins were not detected in ethanol extracts (Table 4). Phytochemical analysis using standard chemical reactions followed by subsequent separation on TLC silica-gel G plates supplemented with suitable detection methods revealed the production of more than four major spots of flavonoids, two spots of iridoid glycosides and two sterols. Alkaloids and saponins were not detected even though more than one investigation method was used.

Table 4: Phytochemical analysis of the *Avicennia marina* shoot system.

| Metabolite                     | Occurrence | Subsequent TLC analysis         | Significance  |
|--------------------------------|------------|---------------------------------|---|
| Volatile oils                  | -          | -                               | Absence from green parts                                      |
| Tannins                        | +          | Hydrolyzable tannins            | Phenolic compound (i.e. may bind to proteins & carbohydrates) |
| Unsaturated sterols & Terpenes | +          | Sterols & terpenes              | Physiologically active growth factors                         |
| Alkaloids                      | -          | -                               | Absence means low toxicity                                    |
| Flavonoids                     | +          | Flavone                         | May have biological activity                                  |
| Glycosides carbohydrates       | +          | Iridoid & flavonoids glycosides | Biologically active glycosides                                |
| Saponins                       | -          | -                               | Absence denotes lack of toxicity                              |
| Organic acids                  | +          | Oxalic & citric                 | Antioxidants  |

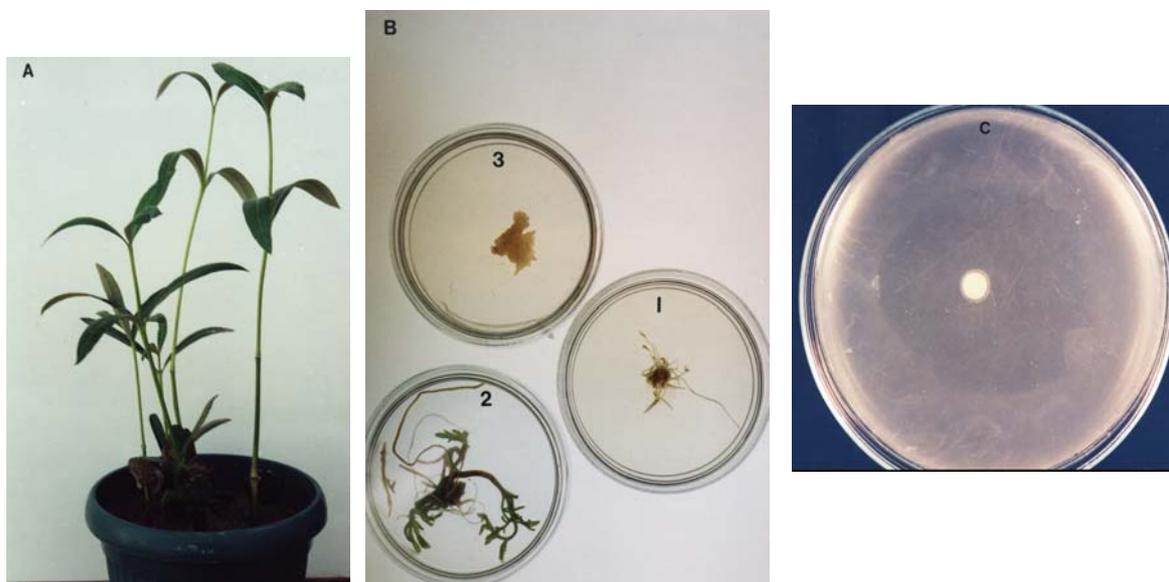


Figure 1: (A) *Avicennia marina* plant materials used for phytochemical screening and biological assays, (B) Carrot hypocotyle *in vitro* assay for growth regulating activity, (C) UV-dependent phototoxic activity of mangrove extracts.

## DISCUSSION

Recent strategies for discovering novel drugs from unexplored natural resources recommended marine plants as an important source of potentially useful chemicals (Harvey 2000). Biological activities and phytochemical screening are essential steps for exploration. Employing a wide range of bioassays (Khafagi *et al.* 2003) is valuable not only for preliminary biological characterization of new compounds, but also as essential guidance of the chemical isolation procedures (Claeson & Bohlim 1997). *Avicennia marina* is used to treat skin diseases in folk medicine, suggesting that it possess some natural antimicrobials. Therefore it was intended not only to test the antimicrobial activity of its extracts (which represents the constitutive activity) but also to test inducible activity elicited by abiotic environmental stresses such as UV radiations (Ferreira & Duke 1997; Khafagi *et al.* 2001, 2003), and anti-bacteriophage and cytotoxic activities.

Our results show that aqueous extracts of both shoots and roots have moderate cytotoxic activities: *in vitro* antimalarial activity and cytotoxicity of *A. marina* have already been reported (Miles *et al.* 1998). The lack of antibacterial activity of various extracts of this plant that grows in an environment occupied by a range of microorganisms was odd as an investigational outcome. Subsequent assays investigating the role of UV radiation to induce immediate production of defence compounds (phytoalexins) verify the formation of instantaneous phytoalexins that are increased as the intensity of the radiation increased. This could be confirmed with the results reported the isolation of a naphthofuran compound with phytoalexin activity (Miles *et al.* 1998). Application of a bioassay to investigate the antibacteriophage activity resulted in the knowledge that *A. marina* extracts have such activity. *Phragmites communis*, known for its value in phytoremediation of wetlands, proved to have antibacteriophage properties associated with its polyphenolic compounds (Tsitsa-Tzardi *et al.* 1990). This is may be understandable as a range of beneficial bacteria are usually found in the sediment of mangrove soils that help promote seed germination and early growth of the seedlings (Holguin *et al.* 2001). *A. marina* may also produce some substances that inhibit the natural enemies of beneficial bacteria (their phages).

Biological activities detected in the plant crude extracts are related mainly to the presence of flavonoids and flavonoid glycosides in plant tissues; the latter have already been isolated from *A. marina*, and have preliminary anticancer activity (Sharaf *et al.* 2000). The presence of compounds such as flavonoids in plant tissues (Winkel-Shirley 2002) may be characteristic features of plant stress.

The absence of toxic metabolites such as alkaloids and saponins from extracts of this plant may reflect its frequent usage as food for local people or feed for their animals. However, the presence of tannins and oxalic acid may predict the unsuitability of such plant for a daily diet.

The presence of plant growth-regulating substances including auxin- and cytokinin-like activities in *A. marina* extracts may be part of normal growth and development. Farrant *et al.* (1993) reported the presence of cytokinins, auxins and gibberellins in the whole fruits of *A. marina* during the histo-differentiation process. Gibberellins are diterpenoids present in the phytochemical content of this plant. Auxins and cytokinins are nitrogenous compounds that are normally produced from amino acids derived from primary metabolism.

In conclusion, *Avicennia marina* plants may have potential medicinal importance. Phytochemical screening shows the presence of compounds that may have biological activity (i.e. flavonoids). The absence of alkaloids and saponins may reflect the low toxicity of this plant. Iridoid glycosides and sterol compounds could be valuable for further

chemotaxonomic studies of the genus *Avicennia*. *In vitro* bioassays using carrot hypocotyls show the presence of growth-regulating substances in the extracts of both seeds and cotyledons, which may help germination and promote plant growth. Recognition of the medicinal and phytochemical value of *A. marina* could enhance conservation plans for mangrove forests in Egypt.

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