# Biological activity of crude extracts of Citrus species from Nigeria

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**Abstract:** The phytotoxicity, cytototixicity and antimicrobial activities of the methanolic, ethyl acetate, dichloromethane (DCM) and hexane extracts of the leaves of *Citrus aurantifolia* and *C. paradisi* were evaluated *in vitro* as part of the screening of ethno-medically useful plants from the Nigerian flora for biological activity. Phytotoxicity using the *Lemna* bioassay in *C. aurantifolia* revealed a significant growth inhibitory effect against *Lemna minor* in DCM, hexane and ethyl acetate extracts at the highest concentrations, and a moderate effect in methanolic fraction. On the other hand, DCM and methanolic extracts from *C. paradisi* had significant effects on *Lemna minor* at the highest concentration, while ethyl acetate and hexane had low activity (20 and 15%), respectively at the highest dose. Cytotoxicity was evaluated using brine shrimp lethality assay and it showed that only the hexane extract from *C. aurantifolia* had an inhibitory effect ($LD_{50}$ 302µg/ml). Also, none of the extracts inhibited the growth of *Escherichia coli, Bacillus subtilis, Shigella flexenari, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Salmonella typhi* bacterial cultures, as well as *Candida albicans, Aspergillus flavus, Microsporum canis, Fusarium solani* and *Canadida glabrata* fungal isolates, thus supporting previous findings on the non-antimicrobial effect of Citrus leaf extracts. The cytotixic and phytotoxic actions in some of the extracts could be considered as a valuable support for the ethno-medicinal uses of the leaves of *C. aurantifolia* and *C. paradisi* in Nigerian folk medicine.
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## 1. Introduction

The sub-genus Citrus (Swingle) comprises of *Citrus aurantium, C. sinensis, C.reticulata* (Tangerine), *C. limon, C. aurantifolia, C. grandis, C. paradisi, C. indica and C. tachibans* etc in the family Rutaceae. *C. aurantifolia* (Christm. et Panz.) Swingle, and *C. paradisi* (Macf.) known as lime and grapefruit respectively, are widely found in Nigeria and many other tropical and subtropical regions (Piccinelli *et al*, 2008). *Citrus* is ranked after banana as the world second fruit crop in production by the volume (more than 108 million tons) (FAO, 2006). Limes are popular for the preparation of juice and carbonated beverages and as a component of alcoholic drinks. Lime juice (100 mL) supplies 110-140 kJ (26 kcal) of energy, 50 mg of ascorbic acid (vitamin C) and traces of dietary fiber (Patil, et al, 2009). Citrus juices are considered to be a rich source of antioxidants, phenolic compounds and carotenoids, beneficial for human health (Patil *et al*, 2009)). Lime leaves have been traditionally used for the treatment of skin diseases and as anti-inflammatory agent. The decoction from leaf is used for eyewash and feverishness by bathing and as a mouthwash and gargle in case of sore throat and thrush (Gattuso *et al,* 2007). Grapefruit is cultivated principally for the juice and has high medicinal value. Its regular use is recommended for building up resistance to common cold and wound infections. In folk medicine, *C. aurantifolia* is used to treat nausea and fainting by squeezing rind near nostril for irritant inhalation. Decoction of roots is used for dysentery and fever and root-bark as febrifuge. The leaves after crushing can be applied to the forehead for headaches. In southwest Nigeria, the root, bark, stem, twig, leaf and fruit are used in the treatment of malaria (Sofowora, 1984).

Lime is commonly known as familiar food and medicine, yet its therapeutic effectiveness in a variety of diseases has been suggested in traditional medicine (Chunlaratthanaphorn *et al*, 2007). Studies on essential oils and antibacterial activity of *C. aurantifolia* have been reported (Dongmo *et a*l, 2009; Negi and Jayaprakasha, 2001; Alanis *et al*, 2005).

The use of *Citrus* flavonoids in cancer treatment has been suggested (Rooprai *et al*, 2001) because of their inhibitory effect on breast cancer cell lines. Although research on the phytotoxicity of plant-based products has increased in recent times, the phytotoxic properties of *C. aurantifolia* and *C. paradisi* have been given little or no attention. Phototoxicity testing has historically used animal models, raising ethical issues. This paper reports the phyto- and cytotoxic and antimicrobial properties of crude extracts of *C. aurantifolia* and *C. paradisi* from Nigeria, using standard bioassay methods.

## 2. Materials and Methods

**Collection and preparation of plant samples**:

Fresh leaves of *Citrus aurantifolia* (Christm.) and *C. paradisi* (Macf.) were obtained from the premises of the University of Ibadan, Ibadan, Nigeria. They were identified at the Department of Botany, University of Ibadan, where voucher specimens of the plants were deposited. The leaves were air-dried in room temperature and later ground into a fine powder. Further processing of the powdered plant materials was carried out at the H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan. The extraction of crude extracts was done by soaking 1.3kg of *C. aurantifolia* and 1.03 kg of and *C. paradisi* in 80% ethanol in a 5- liter beaker each for three days with frequent shaking and filtering until sufficient quantity of the extracts was removed. The solution was stored in a refrigerator at $4^{oC}$ and later concentrated in vacuum with rotary evaporator (Bucchi, Germany), until all the ethanol was evaporated leaving a semi-solid extract.

**Fractionation of crude extracts**:

80g of the crude extract of *C. aurantifolia* was dissolved in 250 ml of hexane solution in a beaker. The mixture was stirred with a glass rod to obtain a homogenous mixture and allowed to settle. Then, the solution was carefully transferred to a 1000 ml separating funnel and allowed to stand overnight undisturbed using a clamp. The aqueous layer was removed, while the supernatant was collected in a beaker. This was carried out until the supernatant became clearer, and kept as hexane fraction. The same process was repeated with dichloromethane (DCM), ethyl acetate and methanol to obtain their respective fractions. For *C. paradisi*, 100g of the crude extract was fractionated as described for *C. aurantifolia* to obtain the individual crude fractions in the same order. All the reagents used were of analytical grade.

**Phytotoxicity**

*In vitro* phytotixicity bioassay was carried out using the modified protocol of McLaughlin, (1991). The *Lemna minor* (duckweed) was cultivated under optimum conditions for 1 to 2 days, briefly washed in water and transferred into the E-medium nutrient (a mixture of various constituents adjusted to pH 5.5-7 to provide nutrients for growth of plant) prior to use. The flasks were initially inoculated with 500, 50 and 5 µg/mL in each of three replicates of the stock solution of the extracts (30 mg of crude dissolved in 1.5 mL MeOH / EtOH). The solvents were left to evaporate overnight and 10 plants of *L. minor*- each containing a rosette of 2 fronds was introduced. Other flasks containing solvent and reference/standard drug paraquat served as negative and positive controls, respectively. The flasks were placed in growth cabinets maintained at 28 ± $1^{oC}$ for 7 days and examined daily. The number of fronds per flask was counted on day 7 to determine the growth inhibition or proliferation of fronds in the flasks.

**Cytotoxicity**

The eggs of brine shrimp *Artemia salina* are available as fish food in pet shops. The eggs were hatched within 48 hr of being placed in artificial sea water. Lethality tests on the extracts were done with initial concentrations of 1:1000 ug $mL^{-1}$ in vials containing 5ml of brine and 10 shrimps in each of three replicates, as described previously (Hopp *et al*, 1996; Onocha *et al*, 2005). Surviving shrimps were counted after 24 hr and the data obtained were processed using a Finney computer programme (McLaughlin, 1991) to obtain the $LD_{50}$ values for each fraction and the standard cytotoxic drug etoposide which served as the positive control.

**Antibacterial activity**

A single colony of *Escherichia coli, Bacillus subtilis, Shigella flexenari, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Salmonella typhi* bacterial cultures obtained from the laboratory stock of ICCBS, University of Karachi, Pakistan were inoculated with nutrient broth and incubate at $37^{o}$ C for 24 hrs. Thereafter, the soft agar tube was removed, melted and cooled to $45^{o}$ C. Then 100 µl of each fresh bacterial culture was added and then poured into plates of nutrient agar and the cultures were evenly spread and allowed to solidify. Wells (6mm-diameter) were made using sterile cork borer and the sample codes indicated in accordance with the bacterial cultures. 100uL of each bacterial sample was added to each agar well plate. Other wells supplemented with DMSO and the reference antibacterial drug imipenum (10 µg/disc) served as negative and positive control, respectively. The plates were incubated at $37^{o}$ C for 24 hrs, and the zone of inhibition in mm were recorded according to the following criteria: - = No activity; 9-12 m = Non

significant; 13-15 mm = Low activity; 16-18 mm = Good activity and above 18 mm = Significant.

**Antifungal activity**

The following fungi species were used as test organisms:*Candida albicans, Aspergillus flavus, Microsporum canis, Fusarium solani* and *Canadida glabrata*. The source is as indicated for bacterial cultures. 24mg of each crude extract was dissolved in 1 ml sterile DMSO to serve as stock solution. The organisms were grown on Sabouraud dextrose agar (SDA) maintained at of pH 5.5-5.6, containing relatively high concentration of glucose or maltose 2% prepared by mixing 32.5 gm/500 ml of sterile water. It was then steamed to dissolve the contents and dispensed as volumes 4ml into screw caps tubes and autoclaved at 121° C for 15 min. The tubes were allowed to cool to 50°C and non-solidified SDA was loaded with 66.6 µl of compound pipette from the stock to give the final concentration of 400 µg/ml of the crude extract. The tubes were then allowed to solidify in slanting position at room temperature. Each tube was inoculated with 4mm diameter piece of fungus removed from a seven-day-old culture. Other media supplemented with DMSO and reference antifungal drug Amphotericin B were used as negative and positive controls, respectively. All the tubes were incubated at 27-29°C for 7 days and examined daily during incubation. Growth in the compound amended media was determined by measuring linear growth (mm) and percentage growth inhibition calculated with reference to the positive control.

**3.Results and Discussion**

There has been an increased interest in the study of *Citrus* plants in the past few years because of the presence of secondary metabolites in different parts of the plant (Piccinelli *et al*, 2008). The current study was focused on the biological activity of leaf extracts from two lime species for possible use as antimicrobial agents. Phytotoxicity using the *Lemna* bioassay in C. *aurantifolia* revealed a significant growth inhibitory effect against *Lemna minor* in dichloromethane (DCM), hexane and ethyl acetate crude extracts at the highest concentrations and a moderate effect in methanolic fraction (Table 1). On the other hand, DCM and methanolic extracts from *C. paradisi* had significant effects on *Lemna minor* at the highest concentration, while ethyl acetate and hexane had low activity (20 and 15%), respectively at the highest dose (Table 1).

**Table 1.** *In vitro* phytotoxicity of *Citrus aurantifolia* and *C. paradisi* leaf extracts

| Citrus species | Extract/Standard drug | Conc. (µg/mL) | No. of fronds in sample[b] | % Growth inhibition |
|---|---|---|---|---|
| *C. aurantifolia* | DCM | 500<br>50<br>5 | 6<br>19<br>20 | 70<br>5<br>0 |
| | Hexane | 500<br>50<br>5 | 5<br>19<br>20 | 75<br>5<br>0 |
| | Ethyl acetate | 500<br>50<br>5 | 8<br>17<br>20 | 60<br>15<br>0 |
| | Methanol | 500<br>50<br>5 | 9<br>20<br>20 | 55<br>0<br>0 |
| *C. paradisi* | DCM | 500<br>50<br>5 | 7<br>19<br>20 | 65<br>5<br>0 |
| | Hexane | 500<br>50<br>5 | 17<br>19<br>20 | 15<br>5<br>0 |
| | Ethyl acetate | 500<br>50<br>5 | 16<br>19<br>20 | 20<br>5<br>0 |
| | Methanol | 500<br>50<br>5 | 8<br>18<br>20 | 60<br>10<br>0 |
| | Paraquat | 0.015 | 0 | 100 |

[b] The sample size is 20 and represents the positive control

**Table 2.** Cytotoxicity (brine shrimp lithality) assay of different extracts of *Citrus aurantifolia* and *C. paradisi*

| *Citrus* species | Extract/Standard drug | Dose (µg/mL) | No. of shrimps | No. of survivors | $LD_{50}$ (µg/L) Brine shrimp lethality |
|---|---|---|---|---|---|
| *C. aurantifolia* | DCM | 1000<br>100<br>10 | 30<br>30<br>30 | 15<br>27<br>29 | 1146 |
| | Hexane | 1000<br>100<br>10 | 30<br>30<br>30 | 10<br>19<br>28 | 302 |
| | Ethyl acetate | 1000<br>100<br>10 | 30<br>30<br>30 | 18<br>25<br>27 | 3695 |
| | Methanol | 1000<br>100<br>10 | 30<br>30<br>30 | 21<br>27<br>29 | 6336 |
| *C. paradisi* | DCM | 1000<br>100<br>10 | 30<br>30<br>30 | 23<br>28<br>29 | 19328 |
| | Hexane | 1000<br>100<br>10 | 30<br>30<br>30 | 22<br>27<br>29 | 10623 |
| | Ethyl acetate | 1000<br>100<br>10 | 30<br>30<br>30 | 20<br>24<br>29 | 3787 |
| | Methanol | 1000<br>100<br>10 | 30<br>30<br>30 | 23<br>27<br>20 | 20682 |
| | Etoposide | | 30 | 0 | 7.4625 |

Cytotoxicity was evaluated using brine shrimp lethality assay and it showed that only the hexane extract from *C. aurantifolia* had an inhibitory effect ($LD_{50}$ 302µg/ml). The rest were found to be non-toxic as they had $LD_{50}$ values greater than 1000µg/ml (Table 2). Also, none of the extracts had antimicrobial effect on all the organisms tested (data not shown). Our finding confirms Ebana *et al*, (1991) who also reported non-negligible antibacterial effect in *C. sinensis* and *C. aurantifolia* against some microorganisms. Most of the effective bioactivity of *Citrus* species reported in literature (Negi *et al*, 2001; Giamperi *et al*, 2004) mostly are from the peels and seeds. *Citrus* peel is reported to be rich in flavanone glycosides and polymethoxyflavones to support its use for industrial application as well as for their pharmacological properties (Ortuño *et al*, 2006 ). Dongmo *et al*, (2009) reported on the antifungal activity of essential oils of three varieties of *C. aurantifolia* (Bearss, Mexican and "Sans épines") from Cameroon. On the other hand, Song *et al*, (2000) attributed the reduced antimicrobial effectiveness of lime to the solubility and volatility of its phytochemical components such as monoterpenes, sesqui-terpenes, oxygenated hydrocarbons, monoterpenes, o-cyme (major component), some of which tend to be lost during the process of extraction especially in organic solvents as explained. Previous reports by Cvetnic and Vladimir-Knezevic (2004) on the antimicrobial activity of *Citrus paradisi*, on *Candida maltosa* and *Cryptococcus neoformans* were negative. The high antimicrobial activity of this plant was attributed to the essential oils, vitamin C and flavonoids that its extracts contained (Cano *et al*, 2008). The cytotixic and phytotoxic actions exhibited in some of the extracts in this study may explain the use of both *C. aurantifolia* and *C. paradisi* in combination with other herbs in folk medicine against malaria fever and other ailments in Nigeria.

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