



Research Article

Phytochemical screening and cytotoxic effects of *Diospyros mespiliformis* Hochst (Ebenaceae) root bark extract using Brine Shrimp (*Artemia salina*) Test

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ABSTRACT

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*Diospyros mespiliformis* belongs to the family Ebenaceae and is used in traditional medicine as an antibacterial, antifungal, and anti-inflammatory agent. This research aims to screen for the phytoconstituents and ascertain the lethal concentration (LC<sub>50</sub>) of the root bark extracts of *Diospyros mespiliformis*. The pulverized sample was extracted with n-hexane, ethyl acetate, ethanol and water in order of increasing polarities with 1.41, 1.44, 8.14 and 4.33 percentage yields obtained respectively. Preliminary phytochemical screening revealed the presence of carbohydrates, flavonoids, saponins, tannins, phenols, steroids/triterpenoids, anthraquinones, alkaloids and cardenolides. The LC<sub>50</sub> of the extracts was found to be greater than 1000 µg/cm<sup>3</sup> suggesting that the extracts contain potent bioactive compounds.

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INTRODUCTION

Natural products derived from medicinal plants have played an important role in drug development, particularly in the treatment of cancer and infectious disorders (Atanasov *et al.*, 2021). Natural products offer special features in comparison with conventional

synthetic molecules as they are cheap to harvest, readily available and have bearable side effects compared to their synthetic counterparts (Ogbiko *et al.*, 2020). Natural products have been reported to exert diverse biological functions such as the control of

endogenous defence mechanisms as well as combat infectious diseases like cancer among others (Atanasov, 2015).

Phytochemicals, also known as phytonutrients notably polyphenols, carotenoids, flavonoids, coumarins, indoles, isoflavones, lignans, organosulfur, catechins, phenolic acids, stilbenoids, isothiocyanates, saponins, procyanidins, phenylpropanoids, anthraquinones, ginsenosides among others are naturally occurring bioactive components found in foods such as fruits, vegetables, whole grain products, nuts and seeds, legumes, tea, and dark chocolate (Xiao, 2017; Singh and Chaudhuri, 2018; Zhao *et al.*, 2018). Several phytochemicals have been extracted and identified from plants, despite the fact; there are tens of thousands of them (Cao *et al.*, 2017).

*Diospyros mespiliformis* commonly called “ebony diospiros” is the largest genus in the Ebenaceae family, having over 500 species widely utilized as a food source in China, Korea, and Japan (Guo and Luo 2011). Locally, the plant is called “kanya”, “burum”, “balchi”, “igi dudu” and “akawayi” by the Hausa, Kanuri, Fulani, Yoruba and Igbo-speaking populace of Nigeria (Usman *et al.*, 2017). The plant is widely distributed throughout Africa, Asia, and parts of Europe. Different part of the plant has been used ethnomedicinal as an antioxidant, hepatoprotective, anti-inflammatory, analgesic, antipyretic, antihypertensive, antidiabetic, neuroprotective, antimicrobial, anti-protozoal, fungicidal, antihelminthic, insecticidal, molluscicidal, cytotoxicity, anti-tumour, multidrug resistance (MDR) reversal, and sedative agents among others (Atta-U-Rahman *et al.*, 2005). The current study aimed to investigate the toxic effects using brine shrimp cytotoxicity as well as to screen for the phytoconstituents present in the various root bark extracts of *D. mespiliformis*.

## MATERIALS AND METHODS

### Plant Material Collection, Preparation and Identification

*D. mespiliformis* plant sample were collected in August 2018 from airport road in the Bodinga Local Government Area of Sokoto State, Nigeria (Long. 5012'26"E, Lat. 12054'59" N). The sample was washed in water to remove earthy impurities before being identified and authenticated at the Botany Unit, Department of Biological Sciences, Usmanu Danfodiyo University Sokoto, Sokoto State, Nigeria. Where a voucher specimen was deposited in the Herbarium, with voucher number UDUSH/ANS/0244. The sample was shade-dried for fourteen days, it was grounded into powder using pestle and mortar stored in an air tight glass jars until ready for use.

### Preparation of plant extracts

Using the Soxhlet extraction method, 500.0 g of the powdered plant material was extracted progressively with 1.5 L solvents of n-hexane, ethyl acetate, ethanol and water in order of increasing polarity for 2 hours. The extracts were concentrated using rotary evaporator at a controlled temperature (40°C – 50°C) using reduced pressure. The extracts were kept in a well-labelled glass jars and stored in the freezer at -4°C. The percentage yield of the n-hexane (NHE), ethyl acetate (EAE), ethanol (ETE), and water (WE) extracts were determined respectively.

### Qualitative phytochemical screening:

Phytochemical screening was carried to identify the phytoconstituents; carbohydrate, alkaloid, flavonoids, tannins, phenols, steroids/triterpenoids, anthraquinones, saponines, and cardenolides in accordance with standard methods (Raaman, 2006; Trease and Evan, 2006; Sofowora, 2008; Nanna, 2013; Silva *et al.*, 2017).

### **Lethality test on *D. mespiliformis* root bark extracts using Brine Shrimp (*Artemia salina*)**

The lethality test of the *D. mespiliformis* root bark extracts were evaluated using Brine Shrimp (*Artemia salina*) nauplii as test organism. The test was carried out in the following manner: hatching of Brine Shrimp, preparation of test solution and Brine Shrimp lethality assay.

#### ***Artemia salina* hatching**

Brine shrimp (*Artemia salina*) eggs were procured locally from Jetunde Foods and Hatchery, Ikeja Lagos State Nigeria. The eggs were hatched using artificial sea water (sea salt (NaCl) solution, maintained at room temperature with constant light illumination. Hatching of the eggs were done according to methods described by (Atta-ur-Rahma *et al.*, 2005). An improvised hatching container maintained at a water height of about 4.00 cm for 24 hours was used. Shrimp larvae with high mobility were used for the experimental bioassay. The nauplii were collected with a Pasteur pipette and kept for an additional 24

hours under the same conditions to reach the metanauplii stage.

#### **Brine Shrimp lethality assay**

Two (2.0) mg/cm<sup>3</sup> of each extract was prepared in 2.0 cm<sup>3</sup> DMSO yielding n-hexane, ethyl acetate, ethanol, and water extracts stock solution. The NHE, EAE, ETE, and WE test solutions were made using the serial dilution method (Parra *et al.*, 2001). 1000 µg/cm<sup>3</sup>, 100 µg/cm<sup>3</sup>, 10 µg/cm<sup>3</sup> and 1µg/cm<sup>3</sup> concentrations were prepared. Using a Pasteur pipette, ten (10) active nauplii were drawn and placed into each test tube containing a different concentration of the test extract, followed by the addition of 2.5 cm<sup>3</sup> of sea water to make the test tube up to 5.0 cm<sup>3</sup>, resulting in concentrations of 1000, 100, 10, and 1 µg/cm<sup>3</sup>. The free medication DMSO was utilized as a negative control, and Vincristine sulphate was employed as a positive control.

The percentage of lethality of the nauplii for each concentration and control was calculated according to Meyer's *et al.*, 1982 as modified by McLaughlin, (1991) and (Clarkson *et al.*, 2004)

$$\% \text{ Death} = \frac{\text{Number of dead nauplii}}{\text{Number of dead nauplii} + \text{Number of live nauplii}}$$

#### **Toxicity testing criteria**

The toxicity of herbal extracts is expressed as LC<sub>50</sub> values that valorized either by comparison to Meyer's or Clarkson's toxicity index. According to Meyer's toxicity index, "extracts with LC<sub>50</sub> < 1000 µg/cm<sup>3</sup> are considered as toxic, while extracts with LC<sub>50</sub> > 1000 µg/cm<sup>3</sup> are considered as non-toxic" (Meyer's *et al.*, 1982 as modified by McLaughlin, 1991).

According to Clarkson's toxicity index, extracts with LC<sub>50</sub> greater than 1000 µg/cm<sup>3</sup> are non-toxic, extracts with LC<sub>50</sub> between 500 and 1000 µg/cm<sup>3</sup> are low toxic, extracts with LC<sub>50</sub> between 100 and 500 µg/cm<sup>3</sup> are medium toxic, and extracts with LC<sub>50</sub> between

0 and 100 µg/cm<sup>3</sup> are very hazardous (Clarkson *et al.*, 2004).

#### **DATA ANALYSIS**

The median lethal concentration LC<sub>50</sub> values of the extracts were determined by plotting percentage death versus the logarithm of the sample concentration (Meyer *et al.*, 1982) using probit regression analysis.

### **RESULTS AND DISCUSSION**

#### **RESULTS**

##### **Brine Shrimp Cytotoxicity**

The brine shrimp lethality activity of the *D. mespiliformis* root bark extracts were shown in Table 1. The n-hexane extract had the highest lethal dose concentration of 8203.52 µg/mL compared to the water extract, which

was 100 % safe at the investigated concentrations. Reference standard vincristine sulfate showed a  $LC_{50}$  value of 1224.56  $\mu\text{g/mL}$ . No mortality was found in the negative control (DMSO) group.

**Table 1: Result of Brine shrimp lethality bioassay**

Extracts	Concentrations ( $\mu\text{g/mL}$ )	Number of nauplii used	Number of surviving nauplii	Mortality (%)	$LC_{50}$ ( $\mu\text{g/mL}$ )
NHE	1000	10	06	40	8203.52
	100	10	08	20	
	10	10	10	0	
EAE	1000	10	04	60	2133.44
	100	10	06	40	
	10	10	09	10	
ETE	1000	10	09	20	2345.22
	100	10	10	0	
	10	10	10	0	
WE	1000	10	10	0	0
	100	10	10	0	
	10	10	10	0	
VS	10	10	0	100	1224.56
	0.50	10	01	90	
DMSO	2%	10	10	0	0

**Key:** NHE- nhexane extract, EAE - ethyl acetate extract, ETE - ethanol extract, WE - water Extract, VS – vincristine sulfate, DMSO – dimethyl sulphur oxide

### Percentage yield

The results of the extractive yield of the extracts are presented in Table 2 using a 500-gram powdered root sample of *D. mespiliformis* after 2 hours of soxhlet extraction. Ethanol has the highest then followed by water ethyl acetate, and n-hexane had the least.

**Table 2:** Percentage yield of the root bark extracts of *D. mespiliformis*

Solvent	weight (g)	Yield (%)	Colour
n-hexane	7.05	1.41	Milky or Milk
Ethylacetate	7.09	1.44	Brownish or Brown
Ethanol	39.5	8.14	blackish or Black
Water	19.34	4.33	brownish or Dark-brown

**Key:** NHE= n-hexane extract, EAE=ethyl acetate extract, ETE= ethanol extract, WE= water extract

### Preliminary Phytochemical Screening

Preliminary phytochemical analyses showed the presence of major classes of secondary metabolite as presented Table 3

**Table 3: Phytochemical constituents of *D. mespiliformis* root bark extracts**

Phytoconstituents	Extracts			
	NHE	EAE	ETE	WE
Flavonoids	-	+	+	+
Saponins	-	-	+	+
Tannins	-	+	+	+
Phenolics	-	+	+	+
Steroids/Triterpenoids	+	+	+	-
Alkaloids	-	+	+	+
Anthraquinones	-	-	-	-
Cardenolides	-	+	+	+
Carbohydrates	-	+	+	+

**Key:** + Present, - Absent

### DISCUSSION

The extractive yields of powdered root samples of *D. mespiliformis* are due to their different polarities which lead to differences in the extractive bioactive compounds (Ogbiko *et al.*, 2020). Ethanol has the highest percentage yield of 8.14, followed by water (4.33), ethyl acetate (1.44) and n-hexane (1.41).

The preliminary phytochemical screening of *D. mespiliformis* root bark (ethanol, ethyl acetate and water) extracts revealed the presence of carbohydrates, flavonoids, saponins, tannins, phenols, steroids/triterpenoids, anthraquinones, alkaloids, and cardenolides. Whereas in n-hexane extract only steroid/triterpenoid was detected. The medicinal value of this is due to their pharmacological activities (Adzu *et al.*, 2003). Phytochemicals have been discovered to have a wide range of functions. Alkaloids, saponins, and tannins, for example, protect against chronic diseases, saponins against hypercholesterolemia, and antibiotic properties, steroids and triterpenoids shows analgesic properties while alkaloids, saponins and tannins are reported to have diverse antimicrobial effects (Kubmarawa, 2007; Mensah, 2008).

The brine shrimp lethality test is an inexpensive, rapid and simple bioassay for

establishing the toxicity of plant extract compared to the animal model. There is a need to screen medicinal plants for their cytotoxic profile because the biological response they bring about is a result of the synergistic effects of their components (Parra *et al.*, 2001; Chanda and Baravalia, 2011; Sharma *et al.*, 2013; Naidu *et al.*, 2014).

The brine shrimp lethality results of *D. mespiliformis* root bark extracts show that the degree of lethality was directly proportional to the concentration of the extract, with the NHE having the highest LC<sub>50</sub> value of 8203.52 µg/mL, the EAE having a value of 2133.52 µg/mL, the ET having a value of 2345.22 µg/mL, and the VS having a value of 1224.56 µg/mL.

Syahmi *et al.* (2010) reported that the LC<sub>50</sub> values produced by the brine shrimp Lethality assay correspond to the LD<sub>50</sub> values obtained by examining the acute toxicity of *E. guineensis* methanol extracts in laboratory Swiss albino mice. Similarly, with Sprague Dawley rats, it was reported that the LD<sub>50</sub> values for acute toxicity of *Mentha spicata* methanol extract evaluated via oral administration are directly related to the LC<sub>50</sub> results obtained by the brine shrimp lethality assay (Naidu *et al.*, 2014).

## CONCLUSION

The cytotoxicity exhibited by the crude extract was promising and this indicates the presence of potent bioactive compounds. This partly justifies the trado-medicinal usefulness of *D. mespiliformis* in treating mild conditions of degenerative diseases associated with free radical damage. Further studies are ongoing to isolate and characterized the active components present in the plant extracts.

## REFERENCES

- Adzu, B., Amos, S., Muazzam, I., Inyang, U.S. and Gamaniel, K.S. (2003). Neuropharmacological Screening of *D. mespiliformis* in mice. *Journal of Ethno-pharmacology*. 83 (1-2). 139-143.
- Atanasov, A. G. (2015). Discovery and resupply of pharmacologically active plant- derived natural products: a review. *Biotechnol. Adv.* 33, 1582–1614.
- Atanasov, A.G., Zotchev, S.B., Dirsch, V.M. (2021). Natural products in drug discovery: advances and opportunities. *Nat Rev Drug Discov* 20,200–216  
<https://doi.org/10.1038/s41573-020-00114-z> PMID: PMC4748402.
- Atta-U-Rahman, M., Iqbal, C. and Thomson, W. J. (2005). Bioassay techniques for Drug Development. 2<sup>nd</sup> Taylor and Francis, Singapore
- Cao, H.T., Chai, X., Wang, M.F.B., Morais-Braga, J.H., Yang, F.C., Wong, R., Wang, H., Yao, J., Cao, L. and Cornara, L. (2017). Phytochemicals from fern species: Potential for medicine applications. *Phytochem Rev.* 16 (3): 379–440. DOI: 10.1007/s11101016-9488-7.
- Chanda, S. and Baravalia, Y. (2011). Brine shrimp cytotoxicity of *Caesalpinia pulcherrima* aerial parts, antimicrobial activity and characterization of isolated active fractions. *Nat. Prod. Res.* 14(3): 34 – 42.
- Clackson, C., Maharaj, V.T., Crouch, N.R., Grace, O.M.Y., Pillay, P., Matsabisa, M.G., Bagwandin, N., Smith, P. J and Folb, P.I., (2004). In vitro antiplasmodial activity of medicinal plant nature to or naturalized in South Africa. *J. Ethnopharm.* 22. 177-191
- Guo, D. L. and Luo, Z. R. (2011). Genetic relationships of the Japanese persimmon *Diospyros kaki* (Ebenaceae) and related species revealed by SSR analysis, *Genet. Mol. Res.* 10 1) 1060–1068.
- Kubmarawa, D., Ajoku, G.A., Enworem, N.M. and Okorie, D.A. (2007). Role of agricultural biotechnology in ensuring adequate food security in developing societies. *Afri. J. Biotechnol.* 6: 1690 – 1696.
- Maharaj, V.T., Crouch, N.R., Grace, O.M.Y., Pillay, P. and Matsabisa, M.G. (2004). In vitro antiplasmodial activity of medicinal plant nature to or naturalized in South Africa. *J. Ethnopharm.* 22. 177-191.
- Mensah, J.K., Okoli, R.I., Ohaju – Obodo, J.O and Eifediyi, K. (2008). Aqueous extract of *Telfairia occidentalis* leaves reduces blood sugar and increases haematological and reproductive indices in male rats. *Afri. J. Biotechnol.*, 7: 2304 – 2309.
- McLaughlin, J.L. (1991). The brine Shrimp lethality bioassay In: Hostettmann, K. Editor, *Methods in plants Biochemistry* London: Academic Press. 8-10.
- Meyer, B.N., Ferrigni, N. R., Putnam, J. F., Jacobsen, L. B., Nicholas, D. E. and McLaughlin, J.L. (1982). Brine Shrimp: A convenient general bioassay for active constituents. *Planta Medica.* 45: 31-34.
- Naidu, J.R., Ismail, R. and Sasidharan, S. (2014). Acute oral toxicity and brine shrimp lethality of methanol extract of *Mentha spicata* L. (Lamiaceae). *Trop J Pharm Res.* 13: 101-107.

- Nanna, R.S., Banda, M., Pemula, P.A. and Kurra, K. (2013). Evaluation of phytochemical and fluorescent analysis of seed and leaf extract of *Cajunu scagam*. *Int. J. Rev. Res.* 22(1):11-18.
- Ogbiko, C., Okoye, F.B.C. and Eboka, C.J. (2020). Phytochemical, in vitro antimicrobial, antioxidant properties and polyphenolic content of methanol leaves extract of *Piliostigma thonningii* Schum Milne-Redhead (Fabaceae). *Afr. J. Pharm. Res. Dev.* 12(3): 299 – 312.
- Parra, L.A., Yhebra, R.S., Sardiñas, I.G. and Buela, L.I. (2001). Comparative study of the assay of *Artemia salina* L. and the estimate of the medium lethal dose (LD<sub>50</sub> value) in mice, to determine oral acute toxicity of plant extracts. *Phyto Med.* 8, 395-400.
- Raaman, N. (2006). Phytochemical techniques. New Indian Publishing Agency. New Delhi. 19-24. 88: 904-910.
- Syahi, A.R.M., Vijayarathna, S., Sasidharan, S., Latha, L.Y., Kwan, Y.P., Lau, L.N. and Chen, Y. (2010). Acute oral toxicity and brine shrimp lethality of *Elaeis guineensis* Jacq. (Oil Palm Leaf) methanol extract. *Molecules.* 15; (11):8111-8121.
- Singh, D., and Chaudhuri, P.K. (2018). A review on phytochemical and pharmacological properties of Holy basil (*Ocimum sanctum* L.). *Industrial Crops and Products.* 118:367– 382. DOI: 10.1016/j.indcrop.2018.03.048.
- Silva, G.O., Abeysundara, A.T. and Aponso, M.M. (2017) Extraction methods, qualitative and quantitative techniques for screening of phytochemical from plant. *American J. Essential Oil and Natural Products.* 5(2): 29-32.
- Sharma, N., Gupta, P.C., Singh, A. and Rao, C.V. (2013). Brine shrimp bioassay of *Pentapetes phoenicea* Linn. and *Ipomoea carnea jacq* leaves. *Der Pharm Lett.* 5, 162-167.
- Sofowora, A. (2008) A medicinal plants and traditional medicine in West Africa. (3th) John Wiley and Sons Ltd. New York. pp: 200 – 203.
- Trease, G.E. and Evans, W.C. (2006). Textbook of Pharmacognosy, 14th. W B Saunders Company Ltd., 24, Oval Road London. pp: 13-53, 117-139, 227.293, 471-511.
- Usman, H., Kura, W.A., Modu, H., Mohammed, B. and Umar, H.A. (2017). Phytochemical and anti-diarrhoeal evaluation of two medicinal plants growing in Maiduguri metropolis Nigeria. *J. Nat. Product and Plant Res.* 7(4): 59 – 64.
- Xiao, J. B. (2017). Dietary flavonoid aglycones and their glycosides: Which show better biological significance? *Critical Reviews in Food Science and Nutrition.* 57:1874–905.
- Zhao, C., Yang, C.F., Liu, B., Lin, L., Sarker, S.D., Nahar, L., Yu, H., Cao, H. and Xiao, J.B. (2018). Bioactive compounds from marine macroalgae and their hypoglycemic benefits. *Trends in Food Science & Technology.* 72: 1–12. DOI: 10.1016/j.tifs.2017.12.001.