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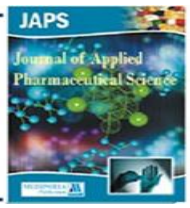

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Cytotoxicity Activity and Phytochemical Screening of *Cochlospermum tinctorium* Perr Ex A. Rich Rhizome

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ABSTRACT

Cytotoxicity activity experiment was carried out on the polar fractions of *Cochlospermum tinctorium* using the brine shrimp lethality bioassay method. The LC_{50} values of the extracts were determined by linear regression analysis method. It was observed that the 80% acetone extract LC_{50} value was 240 ± 3 µg/ ml, which was more potent compared to n-butanol extract with LC_{50} value of 437 ± 8 µg/ ml. Phytochemical test performed on both extracts showed that they contained cardiac glycosides, saponins and carbohydrates, while only the 80% acetone extract contains anthraquinones, flavonoids and tannins. These secondary metabolites present in both extracts, may likely accounts for their cytotoxic activity.

Keywords: Brine shrimp lethality bioassay, cytotoxic, antitumor, Cochlospermum tinctorium, probit analysis, anticancer.

INTRODUCTION

Finding healing powers in plants is an ancient idea. People on all continents have long applied poultices and imbibed infusions of hundreds, if not thousands, of indigenous plants, dating back to prehistory (Cowan, 1999). The search for new drugs which are plant-derived has been receiving renewed interest among researchers throughout the world in view of discovering new drugs that possesses potency to combat the menace of drug resistant pathogenic microorganisms, antitumor and anticancer agents (Mirza, 2007; Pimenta *et al*., 2003). Cancer is the leading cause of death in the United States, where one in four deaths is due to cancer. Recently, there has been difficulty in the treatment of certain forms of cancer due to their resistant to some of the drugs in the market (Mohammed, 2009). Therefore, it has become imperative for natural product chemists to intensify the search for new anticancer and antitumor drugs, which are not only very potent but affordable.

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Among the medicinal plants in the countries of tropical West Africa, *Cochlospermum tinctorium* Perr ex A. Rich is very popular and most traditional herbalist frequently administered different parts of the decoction, infusion, poultices and so on to treat various diseases. *Cochlospermum tinctorium* is called *Rawaya* or *Zunzuna* by the Hausa speaking people in Nigeria and other countries of tropical West Africa. The yellow root or rhizomes are used in many local remedies. Mixed with shea butter and other oils it is applied for burns. A root decoction or infusion is taken with other herbs for stomach troubles and urethral discharges. The Mossi tribe of Cote d' Ivoire use the roots for treating indigestion and stomach pains. It is used in parturition and as an emmenagogue, and the Fulani in Guniea to arrest diarrhea in calves. The root decoction is drunk for orchitis worms and fever in general. It is also used as liniment for epilepsy, pneumonia, intercostals pains, bronchial infections and swelling, and it is said to make a good sitz-bath cure for piles, and to be used for drops for conjunctivitis. The powdered root, in water or millet beer, is supposed to cure jaundice and local application to cure bites of poisonous snakes. It is included with other constituents in local remedies for leprosy (Irvine, 1961; Mann *et al*., 2003). In Northern Cameroon the plant when mixed with other herbs is used to make arrow poison (Castagnou *et al.*, 1965).

Previous research on this plant revealed that the methanol extract of *C. tinctorium* rhizome have antibacterial properties against *Staphylococcus aureus, C. ulcerans, K. pneumoniae, E. coli, Proteus mirabilis* and *Shigelia dysentriae* to give various zone of inhibition values, which was highest with *Staphylococcus aureus* (Tijjani *et al.*, 2009).

The composition of the volatile fractions from *C. tinctorium* rhizome was investigated by GC and GC-MS. Among the 11 constituents detected eight were identified as straight chain ketonic compounds. In addition, five triacylbenzenes were isolated from a petroleum extract of the rhizome, separated by HPLC and identified by their spectral data (Diallo *et al.*, 1991). Two carotenoids cochloxanthine and dihydrocochloxanthine have been isolated from the crude extract of *C. tinctorium* using high speed counter current chromatography (Diallo and Vanhaelen, 1988). Also, two tannins namely – gallic and ellagitaninns have been isolated (Diallo *et al*., 1991).

Furthermore, alkaloids, tannins, cardiac glycosides and flavonoids were detected in the methanol extract of *C. tinctorium* rhizomes (Tijjani *et al*., 2009). In a related development, five compounds were isolated from the dichloromethane extracts of *Cochlospermum tinctorium* rhizome and phytochemical test showed that they are carotenoids and triterpenes (Traoré *et al*., 2006). However, the structures of these isolated compounds have not been fully elucidated.

C. tinctorium have been found to exhibit various kinds of biological activities, which include antiulcer, radical scavenging and immunomodulating activities of the polymers in the aqueous extracts (Nergard *et al*., 2005). Antihepatotoxic actions of the aqueous, ethanol and hydro-ethanol extract (Diallo *et al*., 1987). Antiplasmodial activity of the ethanol extracts of the leaves (Ballin *et al*., 2002; Traoré *et al.*, 2006). Antimalarial activity of the water extracts of the leaves (Benoit *et al.*, 1995). Antifungal and antibacterial activities of petroleum ether extract (Nikiani Ibwala *et al*., 1990).

Based on the aforementioned uses of this plant to cure many diseases, which include respiratory and urinary tract infections and also as a component of arrows poison and its local application to cure bites of poisonous snakes, this paper reports the findings of the brine shrimp lethality bioassay in determining the toxic levels of its polar fractions and phytochemical screening of the fractions in order to determine the secondary metabolites that may be associated with the cytotoxicity. Cytotoxic levels would give an indication about the plants ability as an antitumor agent.

MATERIALS AND METHODS

Collection of plant materials

Rhizomes of *Cochlospermum tinctorium* was collected at the valley behind Ahmadu Bello University Press Limited, Zaria - Nigeria in January, 2010. The plant specimen was authenticated by Mallam U. S. Gallah, Department of Biological Sciences, Faculty of Science, Ahmadu Bello University, Zaria-Nigeria, through comparison with a voucher specimen number 2314 deposited at the herbarium unit of the department.

Extraction of plant materials

The bark of the rhizomes of *C. tinctorium* was removed to reveal the soft yellowish layer. The layer was then cut into pieces and dried immediately at 40 ^{o}C in a laboratory oven to prevent fungal growth because it contains a lot of mucilage. The dried layers were then ground with a mortar to coarse powder. 250 g of the powder was extracted successively with hexane, diethylether, 80% acetone and methanol. The extracts were concentrated in vacuum using a rotary evaporator to give 4.38 g, 5.75 g, 12.2 g and 12.83 g respectively. The 80% acetone solvent used for the extraction was prepared by mixing distilled analytical grade dried acetone with distilled water in the ratio 8:2 that is 8 parts acetone and 2 parts distilled water. 9.0 g of 80% acetone extract was dissolved in 500 ml distilled water and partitioned with 3 x 250 ml each of ethylacetate and n-butanol. The extracts were concentrated in vacuum using a rotary evaporator to give 5.6 g and 2.5 g ethylacetate (EtOAc) and n-butanol (n-BuOH) extracts respectively.

Brine Shrimp Lethality Assay

Sample preparation

0.5 ml of salty sea water was poured into all vials. A control was prepared for each extract being assayed by adding 0.5 ml of sea water and 0.5 ml of dimethyl sulphoxide (DMSO). 80% acetone and n-butanol extracts were the samples examined in this bioassay. Samples were prepared by dissolving 30 mg of each crude extract in 3 ml of DMSO to give stock solutions. Five different concentrations namely 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml and 62.5 µg/ml were prepared in triplicate by serial dilutions from the stock solution. And each concentration

was made up to 3 ml with the salty sea water. A control was prepared using only DMSO.

Hatching the shrimp

Brine shrimp eggs were hatched in a shallow rectangular dish (150 mm x 5 mm) filled with sea water. A plastic divider with several 2 mm holes was clamped in the middle of the dish to make two unequal compartments. The shrimp eggs (*ca* 50 mg) were sprinkled into the larger compartment which was darkened while the smaller compartment was illuminated. After 48 hours the phototropic nauplii were collected using Pastuer pipettes from the illuminated compartment.

Bioassay

Ten larvae of brine shrimps (nauplii) were transferred to each of the prepared vial of different concentration using a Pasteur pipette with a long tip and the total volume in the vial adjusted to 5 ml with sea water. To facilitate easy transfer, nauplii were counted under a magnifying lens (3x) in the stem of the Pasteur pipette against an illuminated background. The vials were maintained under illumination. The shrimps that survived were counted after 24 hours. The total death and percentage mortality (death) at each dose level and control were determined. The results are shown in Tables 1 and 2.

Table. 1: Cytotoxicity of 80% acetone extract of *C. tinctorium.*

Dose (µg/ ml)	Log dose	Total No. shrimps	No. of dead shrimps	% Mortality	Probit
1000	3.000	30	27	90	6.28
500	2.699	30	21	70	5.52
250	2.398	30	15	50	5.00
125	2.097	30	7	23	4.26
62.5	1.796	30	6	20	4.16

Table 2: Cytotoxicity of n-Butanol extract of *C. tinctorium.*

Dose (µg/ ml)	Log dose	Total No. shrimps	No. of dead shrimps	% Mortality	Probit
1000	3.000	30	27	90	6.28
500	2.699	30	12	40	4.75
250	2.398	30	10	33	4.56
125	2.097	30	5	17	4.05
62.5	1.796	30	0	-	-

Statistical analysis

For each extract or sample the lethal concentration that causes 50% death (LC_{50}) was calculated at 95% confidence interval by linear regression analysis. A regression line equation was derived for each extract with the data shown in Tables 1 and 2 respectively, and it was then used to calculate the LC_{50} value. The detailed mathematical steps used to derive the regression line equation are reported in the literature (Finney, 1971; Hubert, 1980; Vincent, 2012). The results are shown in Tables 3.

Table. 3: LC_{50} values of 80% acetone and n-Butanol extracts of *C. tinctorium* brine shrimp cytotoxicity assay.

Extract	Regression line equation	LC_{50} values (µg/ ml)
80% acetone	y = 1.80x + 0.72	240 ± 3
n-Butanol	y = 4.40x - 6.63	437 ± 8

Phytochemical Screening

Phytochemical screening was carried out on both 80% acetone and n-BuOH extracts using standard procedure (Harborne, 1998; Jones and Kinghorn, 2006) to determine the presence of secondary metabolites – namely alkaloids, anthraquinones, cardiac glycosides, flavonoids, tannins, saponins and carbohydrates. The results are shown in Table 4.

Table. 4: Phytochemical screening of polar extracts of C. tinctorium rhizome.

Secondary metabolites	Extracts	
	80% acetone	n-Butanol
Alkaloids	-	-
Anthraquinones	+	-
Cardiac glycosides	+	+
Flavonoids	+	-
Tannins	++	-
Saponins	++	+++
Carbohydrates	++	++

Key: + = present, ++ = high amount present, +++ = extremely high amount present, - = not present.

RESULTS AND DISCUSSION

80% acetone was used in the plant material extraction process in place of pure acetone because generally, aqueous solvents are superior to nonaqueous solvents for extracting glycosides. Water has been found to improve the efficiency of less polar solvents particularly pure acetone. This implies that 80% acetone would extract more components compared to pure acetone (Keinanen, 1993). In terms of components being extracted, 80% acetone extracts mainly glycosides which may be saponins, flavonoids, anthraquinones, and cardiac glycosides (Stahl, 1965). It is important to point out that natural product chemist rarely use acetone for extraction. However, it is an excellent solvent for the extraction of plant polyphenols, especially flavonoids (Cowan, 1999; Afolayan and Meyer, 1997). It was used in this research to not only rekindle its usage but to extract polyphenols in high quantity. However, n-butanol as solvent extracts mainly saponins (Jones and Kinghorn, 2006). Brine shrimp (*Artemia salina* LEACH) is used as a simple bioassay tool for cytotoxicity test on plant extracts. The procedure determines LC_{50} value in µg/ ml of active compounds and extracts in the brine medium. The activities of known active compounds and extracts are manifested as toxicity to shrimps. The advantages of this method are being rapid results, reliability, inexpensive and convenient assay (Meyer *et al.*, 1982). This bioassay has good correlation with cytotoxic activity in some human solid tumors, and has led to the discovery of new class of natural active antitumor agents (McLaughlin *et al.*, 1998).

Table 1 gives the cytotoxicity results for 80% acetone extract while Table 2 for n-butanol extract. However, Table 3 gives the LC_{50} values (median lethal concentration) for 80% acetone and n-butanol (n-BuOH) extracts. The LC_{50} value of n-BuOH is 437 ± 8 µg/ ml whereas that of 80% acetone extract is about 240 ± 3 µg/ ml. Standard brine shrimp lethality bioassay stipulates that an LC_{50} value < 1000 µg/ ml is considered bioactive in toxicity evaluation of plant extracts (Meyer *et al.*, 1982; Parra *et al.*, 2001). Based on this benchmark, the LC_{50} value of 80% acetone and n-BuOH

extracts were both < 500 µg/ ml, this implies that they are highly cytotoxic. Comparison of the LC_{50} values of 80% acetone and n-BuOH extracts as shown in Table 3 indicates that 80% acetone extract is about twice more potent than the n-BuOH extract probably because it contains more active antitumor agents (McLaughlin *et al.*, 1998). The phytochemical test result shown in Table 4 clearly indicates that the 80% acetone extract contains more secondary metabolites with cytotoxic activity compared to n-BuOH extract. This accounts for why its LC_{50} value is about twice more potent.

Saponins are promising anticancer agents that works by stopping cellular mutations that could inevitably lead to cancer (Mohammed *et al.*, 2009). Phytochemical test revealed the presence of cardiac glycosides, saponins and carbohydrates in both 80% acetone and n-BuOH extracts (see Table 4). Liebermann-Burchardt test (Harborne, 1998) indicated that the saponins and cardiac glycosides in both extracts consists of steroidal and terpenoids aglycones. The presence of saponins and terpenes in both extracts justified their potency as antitumor and anticancer agents (Man *et al.*, 2010) based on the LC_{50} values determined from brine shrimp lethality bioassay extracts or compounds that are cytotoxic have good correlation to be effective antitumor agents (Meyer *et al.*, 1982; McLaughlin *et al.*, 1998).

Recently, plant phenolic have demonstrated to be very effective antitumor agents, some of them have been determined to be flavonoids, polyphenols, anthraquinones, coumarins, alkaloids etc. (Lee, 1992). Flavonoids are polyphenolic compounds that are ubiquitously found in plants that accounts for their ability to prevent cancer and tumor growth (Ren *et al*., 2003). Tannins are also polyphenols that have anticancer properties, and it is believed that tannins achieve this property by blocking the production of enzyme required for cancer cell line growth (Mohammed *et al.*, 2006). Only 80% acetone extract contains flavonoids and tannins as shown in Table 4. The presence of these secondary metabolites accounts for the potent cytotoxic activity of the extract. This implies that it may also possess potent anticancer and antitumor activity (Meyer *et al.*, 1982).

CONCLUSION

The brine shrimp lethality bioassay showed that *C. tinctorium* polar extracts are promising antitumor agent based on the LC_{50} values of 80% acetone and n-butanol extracts. The phytochemical test performed on these extracts showed that they contain secondary metabolites which probably account for their cytotoxic activity. Further work needed is to carry out bioassay guided chromatographic separation of each crude extract in order to isolate pure compounds and their structure elucidated by means of spectroscopic analysis like ultraviolet, infrared, mass and nuclear magnetic resonance. In addition, brine shrimp lethality bioassay should be carried out on the pure compounds to determine potent cytotoxic ones and more elaborate cytotoxic bioassay should be performed on the promising potent compounds.

REFERENCES

Afolayan, A.J., Meyer, J.J. The antimicrobial activity of 3,5,7-trihydroxyflavone isolated from the shoots of *Helichrysum aureonitens*. J. Ethnopharmacol. 1997; 57: 177-181.

Ballin, N. Z., Traoré, M., Tinto, H., Sittie, A., Mølgaard, P., Olsen, C. E., Hried, L., Kharazmi, A., Christensen, S. B. Antiplasmodial compounds from *Cochlospermum tinctorium*. J. Nat. Prod. 2002; 65: 1325-1327.

Benoit, F., Valentin, A., Pellisier, Y., Marion, C., Dakugo, Z. P., Mailié, M., and Bastide, J. M. Antimalarial activity *in vitro* of *Cochlospermum tinctorium* tubercle extracts. Transactions of the Royal Society of Tropical Medicine and Hygiene 1995; 89 (2): 217-219.

Castagnou, R., Baudrimout, R., Gauthier, J. Arrow poison used by Fali Tinguelin of Northern Cameroon. Compt. Rend. 1965; 260 (14): 4109.

Cowan, M. M. Plant Products as Antimicrobial Agents. Clin. Microbiol. Rev. 1999; 12 (4): 564-5.

Diallo, B., Vanhaelen, M. Large scale purification of apocarotenoids from *Cochlospermum tinctorium* by countercurrent chromatography. Journal of Liquid Chromatography and Related Technologies. 1988; 11 (1): 227-231.

Diallo, B., Vanhaelen, F. R., Vanhaelen M. Triacylbenzenes and long chain volatile ketones from *Cochlospermum tinctorium* rhizome. Phytochemistry 1991; 30 (12): 4153-4156.

Diallo, B., Vanhaelen, M., Kiso Y., Hikiro, H. Antiheptotoxic actions of *Cochlospermum tinctorium* rhizomes. J. 1987; 20: 239-243.

Finney, D.J. Probit Analysis. Cambridge University Press. Cambridge (1971). ISBN-10: 0521080412, ISBN-13: 978-0521080415.

Harborne, J.B. (1998). Phytochemical Methods: Guide to Modern Techniques of Plant Analysis. Chapman and Hall, London. ISBN-10: 0412572702, ISBN-13: 978-0412572708.

Hubert, J.J. Bioassay. Kendall Hunt Publishing Company (1980).

Irvine, F. R. Woody Plants of Ghana. Oxford University Press, London (1961). pp. 72.

Jones, W.P. and Kinghorn, D.A.. Extraction of Plant Secondary Metabolites From: Methods in Biotechnology, Vol. 20 Natural Product Isolation, 2nd ed. Edited by S.D. Sarker, Z. Latif, and A.I. Gray. Humana Press Inc., Totowa, NJ. (2006) pp. 323-351. eISBN 1-59259-955-9.

Keinanen, M. Comparison of methods for the extraction of flavonoids from Birch leaves (*Betula pendula* Roth) carried out using High-performance liquid chromatography. J. Agric. Food Chem. 1993; 41:1986-1990.

Lee, K.-H. Plant phenolic compounds as cytotoxic antitumor agents. In: Phenolic Compounds in Food and Their Effects on Health II. American Chemical Society Symposium Series Vol. 507 (1992) pp. 367-379. ISBN 9780841224766.

Meyer, B.N., N.R. Ferrigni, Putnam, J.E., Jacobsen, L.B., Nichols, D.E., McLaughlin J.L. Brine Shrimp: A Convenient General Bioassay for Active Plant Constituents. Planta Medica 1982; 45: 31-34.

McLaughlin, J.L.; Rogers, L.L., Anderson, J.E. The Use of Biological assays to Evaluate Botanicals. Drug Information Journal 1998; 32: 513-524.

Man, S., Gao, W., Huang, L., Liu, C. Chemical study and medical application of saponins as anti-cancer agents. Fitoterapia 2010; 81(7): 703-714.

Mann, A., Gbate, M., Umar, A. N. Medicinal and Economic Plants of Nupeland. Jube Evans Books and Publications (2003) pp. 205. ISBN 978-33921-9-0.

Mirza, S.H. Minimising Antibiotic Resistance – Is There a Way Forward? Infectious Diseases Journal Pakistan 2007; 16(3): 75-79.

Mohammed, A., Faruqi, F.B., and Mustafa, J. Edible compounds as antitumor agents. Indian Journal of Science and Technology 2009; 2(5): 62-74.

Mohammed, S. Anticancer agents from medicinal plants. Bangladesh J. Pharmacol. 2006; 1:35-41.

Nergard, C. S., Diallo, D., Inngjerdingen, K., Michaelsen, T. E., Matsumoto, T., Kiyohara, H., Yamada, H., Paulsen, B. S. Medicinal use of *Cochlospermum tinctorium* in Mali, Anti-ulcer, radical scavenging and immunomodulating activites of polymers in the aqueous extracts of the roots. J. Ethnopharmacol. 2005; 96: 255-269.

Nikiani Ibwala, N. Y., Diallo, B., Vanhaelen, M., Vanhaelen, F. R., Pelseneer, J., and Coremans, J. Antifungal and antibacterial activities of two apocarotenoids isolated from *Cochlospermum tinctorium* rhizome. Premier Colloque Européen d'Ethnopharmacologie (1990) 23-25 Mars, ORSTM, pp. 459.

Parra, A.L.; Yhebra, R.S.; Sardinas, I.G., Buela, L.I. Comapartive study of the assay of Artemia salina L. and the estimate of the medium lethal dose (LD_{50} value) in mice, to determine oral acute toxicity of plant extracts. Phytomedicine 2001; 8(5): 395-400.

Pimenta, L.P.S., Pinto, J.A., Takahashi, Silva L.G.F., Boaventura, M.A.D. Biological screening of Annonaceous Brazilian Medicinal plants using *Artemia salina* (Brine Shrimp Test). Phytomedicine 2003;10:209-212

Ren, W., Qiao, Z., Wang, H., Zhu, L., Zhang, L. Flavonoids: Promising Anticancer Agents. Medicinal Research Reviews 2003; 23(4): 519-534.

Stahl, E. Thin layer chromatography; Stahl E. ed. Springer – Verlag, berlin, Heidelberg, New York (1965) pp. 690-691.

Tijjani, M. B., Bello, I. A., Aliyu, A. B., Olurishe, T., Maidawa, S. M., Habila, J. D., Balogun, E. O. Phytochemical and Antibacterial Studies of Root Extract of *Cochlospermum tinctorium* A. Rich (Cochlospermaceae). Res. J. Med. Plant 2009; 3(1): 16-22.

Traoré, M., Guiguemdé, A., Yago, I., Nikièma, J. B., Tinto, N., Dakuyo, Z. P., Ouédraogo, J. B., Guissou, I. P., and Guiguemdé, T. R. Investigation of antiplasmodial compounds from two plants, *Cochlospermum tinctorium* A. Rich and *Gardenia sokotensis* Hutch. Afr. J of Trad. CAM 2006; 3(4): 34-41.

Vincent, K. Probit Analysis. http://userwww.sfsu.edu/~efc/ classes/ biol710/probit/ProbitAnalysis.pdf as retrieved on 15/05/2012