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Phytochemical Composition and Brine Shrimp Cytotoxicity Effect of *Rosmarinus officinalis*

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Abstract

Plant compounds exhibit enormous structural diversity, unfortunately only a small proportion of that diversity has been seriously explored for pharmacological potential. The use and commercialization of non-timber plant products which include medicinal plants has been found to be an important livelihood strategy in developing countries especially for the rural people. The current study was carried out on the crude aqueous extracts of leaves of *Rosmarinus officinalis* (rosemary) to evaluate the plants phytochemical composition using standard methods. The cytotoxicity and lethality effects on the brine shrimp (*Artemia salina*) of four organic extracts and also an aqueous extract of the rosemary leaves was studied using three concentrations (10, 100, 1000) µg/ml. The data was analyzed using Finney's probit analysis method with the help of Biostat 2009. The phytochemical analysis showed presence of; terpenoids, tannins, cardiac glycosides, flavonoids, reducing sugars and saponins. All the extracts gave moderate medial lethal concentration (LC_{50}) between 220 and 470 µg/ml. Cytogenic compounds in the extract caused the brine shrimp high lethality which corroborates the wide use of rosemary in the health care. Rosemary plant could be seen as a good source for useful drugs.

Keywords: *Artemia salina;* bioactive; cytogenic; extract; lethal concentration; mortality

1. Introduction

Medicinal plants are gaining an increasing attention in solving problems in the health sector. There is a resurgence of interest in the research on plants of medicinal importance internationally. Medicinal plants are the

richest bio resources of folk medicine, food supplement, nutraceutical pharmaceutical and chemical entities of synthetic drugs [1]. The market and public demand of herbal medicine have been increasing due to belief that they are less toxic consequently leading to an increased risk of extinction or loss of genetic diversity [2].

Kenya has a rich plant heritage with very potent biochemicals [3, 4]. Like many other developing countries, the nation, has a wide spread use of medicinal plants especially in the rural areas [5]. The term phytochemicals is generally used to refer to components that may have biological significance for example, the antioxidant [6]. There are over 10,000 different phytochemicals having the therapeutic effects against cancer, stroke or metabolic syndrome [7]. Phyotomedicine can be derived from barks, leaves, flowers, roots, fruits, seeds [8]. Knowledge of the phytochemical constituents of plants is desirable because such information will be of value in the synthesis of complex chemical substances [9]

The use and commercialization of non-timber forest products which include medicinal plants has been found to be an important livelihood strategy in developing countries especially for the rural people [10], hence enhancing their living standards [11]. The brine shrimp lethality bioassay is considered a useful tool for preliminary assessment of cytotoxicity of medicinal plants [12] eventually leading to isolation of bioactive compounds from plant extracts [13]. This method is very simple and inexpensive. The present work studies the brine shrimp cytotoxicity of four organic extracts and an aqueous extract of the dried leaves of *Rosemarinus officinalis.*

R.officinalis (rosemary) belongs to the *Lamiaeceae* family and is an aromatic, evergreen, shrubby herb and grows to a height of up to 2m. This plant is a native to the Mediterranean region and derives its name from its refreshing effects. Rosemary is widely used and grown all over the world as a decorative plant gardens or even as a fence and has many culinary and medical uses. *R. officinalis* is widely used as a spice when cooking, especially the Mediterranean dishes and it has naturally occurring antioxidant [14]. Other traditional medicinal use of rosemary includes: relieve muscle pain and spasm ,stimulate hair growth, stimulate circulatory and nervous system, increase urine flow ,treat indigestion ,relieve of respiratory disorders, as an analgesic, antirheumatic and antiepileptic [15].

Studies have shown that rosemary plant due to its dilatory properties can increase blood flow and its external use has vasodilatory effects on the skin [16]. Rosemary has also been found to have food preservative qualities [17]. *Rosmarinus officinalis* essential oils or some, of their components are commonly used in make-ups, and sanitary products [18].

2. Materials and methods

2.1. Plant materials

Rosmarinus officinalis was kindly provided by “Tarcit Energies”, a farm in Meru area of Kenya which produces and sells rosemary. Identification and authentication of the plant was done at the herbarium, School of Biological Sciences, University of Nairobi.

2.2.1. Extract preparation

The leaves of *R. officinalis* were cleaned with tap water and rinsed with distilled water, air-dried at room temperature (22-26°C) to a constant weight after which they were ground to a uniform powder using an electric mill. The powder (100g) was soaked in 1000ml of deionized water for 48 hours after which it was filtered using cotton wool and also whatman filter paper.

The filtrate was frozen for 24 hours after which it was lyophilized in a freeze drier to get a dry powder (6.2g) which was stored in air-tight bags and stored at 4^{o}C.

2.2. Phytochemical screening

Phytochemical screening was performed using [19].

2.2.1 Test for anthraquinones

A sample (0.5g) of the aqueous extract was boiled with 10 ml of sulphuric acid, H_2SO_4, and filtered while hot. The filtrate was shaken with 5 ml of chloroform and the chloroform layer pipetted into another test tube after which 1 ml of dilute ammonia was added. The resulting solution was observed for colour changes. A yellow colour indicated presence of anthraquinones.

2.2.2. Test for terpenoids (Salkowski test)

To a 0.5g sample of the extract was added 2 ml of chloroform. Concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface indicated the presence of terpenoids.

2.2.3. Test for flavonoids

Dilute ammonia (5 ml) was added to a portion of an aqueous extract. Concentrated sulphuric acid (1 ml) was also added. A yellow colouration that disappeared on standing indicated the presence of flavonoids. A second method entailed adding a few drops of 1% Aluminum a portion of the filtrate.

A yellow colouration indicated the presence of flavonoids. The third a portion of the extract was heated with 10 ml of ethyl acetate over a steam bath for 3 minutes. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution and observed for a yellow colouration.

2.2.4 Test for reducing sugars (Fehling's test)

Equal volume of Fehling A and Fehling B reagents were mixed together. A 2 ml portion of the mixture was added 1ml of crude extract solution of the plant and gently boiled. A brick red precipitate appeared at the bottom of the test tube that indicated the presence of reducing sugars.

2.2.5 Test for saponins

To a 0.5 g of extract for was added 5 ml of distilled water in a test tube. The solution was shaken and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion.

2.2.6 Test for tannins

About 0.5 g of the extract was boiled in 10 ml of water in a test tube and then filtered.

A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration which was positive for tannins.

2.2.7 Test for alkaloids

About 0.5 g of extract was diluted to 10 ml with acid alcohol, boiled and filtered. To 5 ml of the filtrate was added 2 ml of dilute ammonia. Chloroform (5ml) was then added and shaken to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Draggendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggendorff's reagent) was regarded as positive for the presence of alkaloids.

2.2.8. Test for cardiac glycosides (Keller-Killiani test)

A sample of the extract (0.5g) was mixed with 5 ml distilled water and 2 ml of glacial acetic acid containing one drop of ferric chloride solution was added. This was underplayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides.

A violet ring in some cases appeared below the brown ring, while in the acetic acid layer a greenish ring formed just above the brown ring and gradually spread throughout these layers. This indicated presence of cardiac glycosides

2.3 Brine shrimp lethality bioassay

2.3.1 Extract preparations for brine shrimp assay

Four organic and one aqueous extracts were used for this assay. The organic solvents used were; Hexane, DCM (Diclomethane), DCM: methanol (1:1). Methanol: water (95:5). *R.officinalis* (50g) powder was soaked in 500ml of each of the solvents for 48 hours, filtered using cotton wool and No.1 Watman filter paper. The filtrates were evaporated in a rotor –evaporator at 40^{o}C to complete dryness. The aqueous extract was freeze dried. These extracts were stored in air tight containers at 4^{0} C.

2.3.2. Culturing and harvesting of A. salina

Artemia salina (2g) cysts were incubated for hatching in a shallow rectangular dish with a plastic divider with several 2 mm holes making two unequal compartments. The container was filled with 3.3% solution of artificial sea water and 50 mg yeast sprinkled into the larger compartment which was darkened. The smaller compartment was illuminated by a tungsten filament light and gently sparged with air. After 24 hours, hatched *A. salina* cysts were transferred to fresh artificial seawater and incubated for a further 24 hours under artificial light. The phototropic nauplii were collected by pipette from the lighted side.

2.3.3. Preparation of test extracts and the controls

Stock solutions of for each of the organic extracts (10 000 µg/ml) was made in dimethyl sulphoxide (DMSO)

Extracts aliquots of 5 µl, 50 µl, and 500 µl for 10 µg/ml, 100 µg/ml, and 1000 µg/ml respectively, was transferred into 15 ml tubes and made to 4.0 ml with the brine solution. All tests were done in triplicates.

2.3.4. Brine shrimps assay.

A. salina nauplii (10*)* were counted macroscopically in the stem of a Pasteur pipette against a lighted background and transferred into each sample vial and the solutions were made to 5ml with brine solution. A drop of dry yeast suspension was added as food to each vial. All the vials were maintained under illumination. The surviving nauplii were counted with the aid of a 3x magnifying glass after 24 hours. The mean mortality at the three dose levels for each extract was determined. The surviving nauplii were killed by the addition of 100 µl of 5% (v/v) phenol to each tube.

2.3.5. Statistical analysis to determine LC_{50}

Data was analyzed by Finney's probit analysis method with the help of Biostat 2009. Probit analysis is a method of survival analysis which involves conversion of the percentage mortalities into probits and also conversion of the concentrations into logarithms. Plotting the logarithms against the probits gives a straight line which can be used to precisely estimate medial lethal dose or concentration (LD_{50}/LC_{50}).

3. Results

3.1 Phytochemical screening

The phytochemical assays showed the presence of; terpenoids, tannins, cardiac glycosides, flavonoids, reducing sugars and saponins in the rosemary extract. Alkaloids and anthraquinones were absent in the extract, **(table 1).**

3.2 Brine shrimp assay

All the five types of extracts showed mortality of brine shrimp.

Total mortality was observed in 1000 µg/ml concentration and no brine shrimp larvae was killed in the lowest concentration (10 µg/ml) in all extracts**, (table 2).** All the extracts showed moderate medial lethality concentration between 200 and 500 µg/ml. extract had the lowest LC_{50} of 221.51±164.99.

Table 1: Phytochemical assay results *of R.officinalis* crude aqueous extracts.

Test for	results
Terpenoids –Salkowish test	+ve
Tannins	
-Lead sub acetate test	+ve
-Ferric chloride test	+ve
Cardiac glycosides	+ve
Anthraquinones glycosides	-ve
Flavonoids	
-Ammonium test	+ve
-Aluminum chloride test	+ve
Saponins (Frothing test)	+ve
Reducing sugars	+ve
Alkaloids	
-Dragerndroff test	-ve
-Mayers test	-ve
-Wagner test	-ve

Key:-ve and +ve sign indicate absence or presence of the phytochemical respectively.

Table 2*:* Average mortality of Brine shrimp larvae out of 10 at various concentrations and their estimated LC_{50} value ± standard error (SE) for the five extracts of *Rosmarinus officinalis*

Extract	Average mortality in			LC_{50}(µg/ml)	SE
	1000 (µg/ml)	100 (µg/ml)	10 (µg/ml)		
Hexane	10	0	0	470.13	±119.32
DCM	10	5.67	0	221.51	±164.99
DCM: Methanol	10	1	0	404.04	±131.46
Methanol: Water	10	0	0	470.13	±119.32
Water	10	0	0	470.13	±119.32

4. Discussion

Therapeutic property of medicinal plants is believed to be due to the presence of various secondary metabolites. Rosemary is an important plant worldwide used traditionally for health care benefits. The data generated from

the phytochemical investigations on the crude aqueous extract suggests that terpenoids, tannins, saponins, flavonoids, cardiac glycosides and reducing sugar present are the active constituents in the dry leaves extract of rosemary. The study showed the absence of alkaloids and anthraquinones glycosides (table 1).

Toxicity studies also are important in identification and also isolation of new compounds from crude extracts [20]. In this study solvents with different polarity ranges were used in order to study the cytotoxicity of the bioactive compounds of rosemary leaves extracted in each solvent. The activities of the extracts are manifested as toxicity to shrimps by bioactive components present in the extracts. A substance is considered to be cytotoxic if it inhibits vital metabolic processes or it causes disorders in living organisms resulting in perversion of behavior or death [21]. Standard brine shrimp lethality bioassay stipulates that an LC_{50} value < 1000 µg/ ml is considered bioactive in toxicity evaluation of plant extracts [12].The lethality of extracts to the brine shrimp larvae was found to be directly proportional to the concentration of the extract. Total mortalities took place at a concentration of 1000 µg/ml in all the five extracts and no mortality that occurred at 10 µg/ml. All the extracts gave moderate LC_{50} (less than 500 µg/ml), table 2.

Different solvent extracted different bioactive components. Hexane, for instance, extracts waxes, fats, and fixed oils [22]. The study suggests the presence of these compounds in the hexane extract of *R.officinalis* dried leaves. Diclomethane is a more polar solvent and is commonly known to extract alkaloids, aglycones, and volatile oils [22]. Rosemary essential oils are of great interest due to their pharmacological properties [23]. Essential oils are composed of different mixtures of volatile compounds usually with strong odor and they exert diverse ecological functions [24].The report suggested that rosemary essential oils were extracted into this solvent and could have contributed to the high mortality of the brine shrimps.

Combining methanol and DCM seemed to have more cytogenetic effects than the *95%* methanol which was demonstrated by the lower LC_{50} (404.04) µg/ ml, table 2. Aqueous extract *of R.officinalis* gave an LC_{50} of 470 µg/ ml, which showed that water is able to extract cytogenic bioactive components, validating why rosemary water infusion is commonly taken as herbal tea for its therapeutic property [15].

5. Conclusion

The study suggests that the brine shrimp lethality was contributed by the phytochemical composition that was extracted by the various extraction solvents. The moderate LC_{50} of less than 500µg/ ml corroborates the wide use of rosemary as therapeutic agent. All the five types of extracts contained biologically active compounds and therefore, extracts from rosemary plant could be seen as a good source for useful drugs.

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References

[1] N.S Ncube, A.J Afolayan and A.L Okoh**,** "Assessment techniques of antimicrobial properties of natural compounds of plant origin: Current methods and future trends". *African Journal of Biotechnology*, Vol7:1797-1806, 2008.

[2] A.Misra**. "**Studies on biochemical and physiological aspects in relations to phyotomedicine handling, cultivation and harvesting of the medicinal plants". *Journal of Medicinal Plants Research* Vol**.**3, pp. 1140-1146, 2009.

[3] J.O Kokwaro. *Medicinal plants of East Africa*, Second edition, Kenya Literature Bureau. Nairobi, 1993 pp. 401

[4] W.Riley and D.Brokensha**.** *The Mbeere in Kenya: Botanical identities and uses*, Vol. II University Press of America, Lanham, 1988, pp: 80-91.

[5] J.C Tilburt and T.J Kaptchuk,. *Herbal medicine research and global health*: An ethical analysis Bulletin of the World Health Organization 86 577–656, 2008.

[6] **S.** Agarwal, A.V Rao. **"**Lycopene content of tomato products, its stability, bioavailability and in vivo antioxidant properties. *Journal of medicinal food* **4 (1):** 9–15,2001

[7] J.Higdon**.** *In an Evidence-Based Approach to Dietary Phytochemicals*. First edition New York, 2007, pp 155-161

[8] G.M Criagg and J.N David**. "**Natural products of some Nigerian Medicinal Plants". *African Journal of Biotechnology* , vol **4:** 685-6885, 2001

[9] J. Parekh and S. Chanda**. "**Antibacterial and phytochemical studies on twelve species of Indian medicinal plants". *African journal of Biomedical Research*, **10**: 175-181, 2007.

[10] D.Mbuvi. and E Boon. "The livelihood potential of non-wood forest products: The case of Mbooni Division in Makueni District, Kenya. Environment Development and Sustainability" Volume **11(5):**989-1004,2007

[11] C.M Shackleton, E Parkin, M.LChauke, L. Downsborough, A. Olsen .G. Brill and C. Weideman**. "**Conservation, Commercialization and Confusion: Harvesting of *Ischyrolepis* in a coastal forest, South Africa. Environment Development and Sustainability" **11**:229-240, 2009.

[12] B.N Meyer, N.R Ferrigni, J.EPutnam , L.B Jacobson, D.ENichols and J.L Maclaughlin**.** "Brine shrimp, a convenient general bioassay for active plant constituents". *Planta Medica*, 45: 31-34, 1982.

[13] T.W Sam. *Toxicity testing using the brine shrimp: Artemia salina. In: Colegate S.M. and Molyneux, R.J. (Editions.), bioactive natural products detection, isolation and structural determination*'. CRCPress, Boca Raton, Fl, 1993, pp: 442-456.

[14] R. Inatani, N.Nakatani and H.Fuwa. "Antioxidative effect of the constituents of rosemary (Rosemarinus officinalis L) and their derivatives". *Agriculture, Biology and Chemistry* 47: 521–528 1983.

[15] M. Blumenthal, A. Goldberg and J. Brinckmann**.** *Herbal Medicine: Expanded Commission E Monographs. Newton, MA: Integrative Medicine Communications*,2000 pp 326-32956

[16] W.H Frishman, S.T Sinatra, M. Moizuddin**.** "The use of herbs for treating cardiovascular disease". *Seminars in Integrative Medicine*"vol. 2: 23-35,2004.

[17] S.O Oiye, S. Konoye, S.Ngala **. "**Effects of Rosemary, (*Rosemarinus Officinalis L.*) Spice and Nitrite Picking Salt Combination on Keeping and Organoleptic Quality of Beef Sausages*"*. *Journal of Basic of Scientific Research* ,**2(4)**: 4008-4015,2013

[18] T.U Bakirel, O.U,Keles., S.GUlgen and H.Yardibi**.**"In vivo assessment of antidiabetic and antioxidant activities of rosemary (Rosmarinus officinalis) in alloxan-diabetic rabbits*"*. *Journal of Ethnopharmacology, 116*(1):64-73, 2008.

[19] J.B. Harborne. *Phytochemical methods: A guide to modern techniques to plant analysis.* 2nd edition. London: Champman and Hall, 1988: pp. 55-56

[20] S**.** Sasidharan , I. Darah, K. Jain. "In vivo and in vitro toxicity study of Gracilaria changii." *Pharmaceutical Biology*. 46(6):413–417, 2008.

[21] M.O Fatope**.** "Phyto-compounds, their bioassay and diversity, discovery and innovation, food in O.Aruoma and B.Halliwell editions. *Free Radicals and Food Additives*, London: Taylor and Francis Ltd., 1995:pp. 77-119

[22] M. Cowan. "Plant products as antimicrobial agents". *Clinical Microbiology Reviews* 12 (4): 564-582

[23] A. Kadri, Z. Zarai Z, I. B Chobba, A. Bekir, N.Gharsallah and M.Damak, R. Gdoura**."** Chemical and antioxidant properties of *Rosmarinus officinalis* L. essential oil cultivated from South-Western Tunisia" .*Journal of Medicinal Plants Research*, 5:5999-6004, 2011

[24] Teixeira, A. Marques, C. Ramos, N.R Neng, JMF. Nogueira, J.A Saraiva and M.L Nunes. "Chemical composition and antibacterial and antioxidant properties of commercial essential oils". *Indian Crop Production*, 43:587-595, 2013.