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Histological Evaluation, Antioxidant and Cytotoxic Studies of Miliusa wayanadica

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Abstract:
Aim of the Study: *The present study describes the morphology and anatomy of Miliusa wayanadica leaf, Evaluates antioxidant activity and in vitro cytotoxic activity of the leaf extracts by in-vitro models.*
Method: *Anatomical sections of the leaf were observed by microscopic evaluation. Histochemical tests were conducted on ethanolic leaf extracts to determine the presence of various constituents in the sample. Antioxidant activity of the leaf extracts were done using in vitro methods DPPH, FRAP and nitric oxide scavenging assay. The cytotoxic assay done by using DLA & EAC cell lines and also by using brine shrimp bio assay method*
Result: *Transverse sections of the leaf were observed by microscopy. Different radical scavenging assays showed prominent antioxidant activity and also potent cytotoxic effect*
Conclusion*: The evaluation of antioxidant activity& cytotoxic effect of Miliusa wayanadica conclude that the plant extracts showed the ability and may be due to the presence of flavonoids and phenols*
Key words: *Transverse section, phytochemical analysis, Phenol, Flavonoid, Antioxidant activity, cytotoxic activity, Miliusa wayanadica (Annonaceae)*

I. Introduction

In the plant kingdom the Annonaceae is a large family comprising of about 135 genera and 2500 species and is widely spread along the tropical and sub-tropical regions. Species within the family are known to contain terpenoids (monoterpenes and diterpenes), alkaloids, acetogenins and flavonoids. These secondary metabolites have been found to have many biological activities which include anti tumor, antiviral, phytotoxicity, hormonal actions, cardio-protective actions, antioxidant effects and free radical scavenging activities.

The genus Miliusa has over 40 different species spread over the Austral-Asiatic continent. The species Miliusa wayanadica was recently discovered at Kurichianmala and Kalpata in Wayanad District along the Western Ghats. These shrubs are seen in evergreen forests growing along the sides of streams. However, there are very few studies reported on the genus for its morphology, phytochemical constituents and antioxidant studies. The main goal of the present study is to characterize the morphology of the leaf as well as to the phytochemical constituents and explore the potential in vitro antioxidant properties of leaf extract of Miliusa wayanadica. [4]

II. Materials & Methods

Plant materials

The plant was collected from the Western Ghat of Kerala during the month of December. It was then biologically authenticated by taxonomist Dr. M.K Ratheesh Narayanan, senior scientist, M S Swaminathan research foundation, Wayanad.

Microscopic studies

Thin sections of fresh leaves were obtained by free hand sectioning. Toludine blue was used as stain and the transverse section was observed under Leica DM 1000 microscopy.

Preparation of extract

The coarse powder (50 gm) was extracted using ethanol as a solvent. The resulting solvent were removed by reduced pressure and resulting semi solid was vacuum dried using rotary flash evaporator to get a solid residue and named it as ethanolic extract of Miliusa wayanadica . The dried extract obtained was used to determine the antioxidant activity

Preliminary Phytochemical Analysis

The dried ethanolic extracts of Miliusa wayanadica were used to analyse various phytochemicals like alkaloids, proteins, steroids, saponins, flavonoids, phenolic compounds and tannins. Phenolics: 2 ml of filtrate + 2 ml Ferric chloride, blue precipitate indicated presence of phenolics. Saponins (frothing test): 0.5 ml filtrate +5 ml distilled water; frothing persistence indicated presence of saponins. Alkaloids: 2 ml of filtrate +1% hydrochloride + Dragendroff reagent, orange precipitate indicates the presence of alkaloids. Flavonoids; 5 ml dilute ammonia was added to a portion of filtrate + concentrated sulphuric acid; yellow colour indicates presence of flavonoids. [14, 17]

Determination of Fluorescence Analysis

Powdered dried leaves was subjected to analysis under ultra violet light after treatment with various chemical and organic reagents

Total Phenolic Contents

Total phenolic contents (TPC) were measured calorimetrically using the Folin-Ciocalteau method. 1ml of appropriately diluted leaf extract (using 80% ethanol) of Miliusa wayanadica was taken. 2ml of 20% Na_2CO_3 solution and 0.5 ml Folin-Ciocalteau phenol reagent were added to the sample and shaken vigorously. The absorbance was then measured at 765 nm. Gallic acid is taken as standard.

Total Flavonoid Content

Total flavonoid content was determined using Aluminium chloride calorimetric method. Plant extract was dissolved in 80% ethanol to make a final concentration of 1mg/ml. Then 0.5ml of the extract was mixed with 0.3ml of 5% Sodium nitrate and incubate for 5mins. Add 6ml of 2%aluminium chloride to the mixture and incubate for another 5mins. The procedure is repeated for standard quercetin. The absorbance is measured at 512nm.

DPPH Free Radical Scavenging Activity

The radical scavenging activity of the dried extract was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH(2,2-diphenyl-1- picrylhydrazyl [2,16]. 0.1 mM solution of DPPH in ethanol was prepared; 1 ml of the solution was added to 2.5 ml of extract in ethanol at different concentrations (100-500 µg/ml). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm by using a UV-Visible Spectrophotometer (Schimadzu UV-Visible 1700) and compared with BHT and BHA as the standard. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. [17-20].The percentage DPPH scavenging effect was calculated using the following equation:

DPPH scavenging effect (%) = $((A_0 - A_1)/ A_0) \times 100$

Where, A_0 is the absorbance of control at 517 nm;
A_1 is the absorbance of sample extract/standard at 517 nm.
Tests were performed in triplicate and the results were averaged [19]

Nitric Oxide Scavenging Assay

Nitric oxide scavenging activity was measured spectrophotometrically. Extract prepared in ethanol, was added to different test-tubes in varying concentration (0.5, 1, 1.5, 2, 2.5 mg/ml). Sodium nitroprusside (5mM) in phosphate buffer was added to each test tube to make volume up 1.5 ml of Griess reagent (1% sulphanilamide, 0.1%naphthylethylenediamine dichloride and 3% phosphoric acid) was added to each test tube. The absorbance was measured, immediately, at 546 nm percentage of scavenging activity was measured with reference to ascorbic acid standard. [15]

Ferric Reducing Antioxidant Power (FRAP) Test

The FRAP assay was done according to Benzie and Strain (1996). The stock solutions included 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mMHCl, and 20 mM $FeCl_3$.6H2O solution. The working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3.6H_2O$ solution and then warmed at 37 C before using. 200 µl of leaf extract was added to 3 ml of the FRAP reagent. After incubation in the dark at 37°C for 30 min, the colored product [ferrous tripyridyltriazine complex] was then read at 593 nm. [16]

In Vitro Cytotoxicity Study Using DLA & EAC Cell Lines

The test compounds were studied for short term in vitro cytotoxicity using Dalton's lymphoma ascites cells (DLA) or Ehrlich Ascites Carcinoma (EAC) cells.

The tumour cells aspirated from the peritoneal cavity of tumour bearing mice were washed thrice with PBS or normal saline. Cell viability was determined by trypan blue exclusion method. Viable cell suspension was added to tubes containing various concentrations of the test compounds and the volume was made up to 1 ml using phosphate buffered saline (PBS). Control tube contained only one cell suspension. These assay mixture were incubated for 3 hour at 37`c. Further cell suspension was mixed with 0.1 of 1% trypan blue and kept for 2-3 minutes and loaded on a haemocytometer. Dead cells take up the blue colour of trypan blue while live cells do not take up the dye. The number of stained and unstained cells was counted separately. [22]

III. Brine Shrimp Cytotoxicity

Materials

Brine shrimp (Artemia Salina) eggs, Dram vials, Petri dish

Methods

Sample preparation: Samples were prepared by dissolving 5mg of extract in 5ml of 5% ethanol. Appropriate amount of solutions were transferred to 0.5cm paper discs were dried in 2-dram vials and then dried further in vacuum for one hour. Control discs were prepared using ethanol in water. Three replicates were prepared for each dose level. [17]

Hatching the shrimp

Brine shrimp eggs (Central Fisheries Research Institute Kochi) were hatched in a shallow rectangular dish (22 x 32 cm) filled with artificial sea water, which was prepared with a commercial salt mixture and distilled water. A plastic divider with several 2mm holes was clamped in the dish to make two unequal compartments. The eggs (50mg) were sprinkled on top of the larger compartments, which was darkened while the smaller compartment was illuminated. After 48 hours the phototropic naupili were collected using pipette from the lighted side, having been separated by the divider from their shells. [17]

Artificial sea water has the following composition

Ingredients	**grams/liter**
Sodium chloride	28.566
Magnesium chloride	3.887
Magnesium sulphate	1.787
Calcium sulphate	1.308
Potassium hydrogen sulphate	0.832
Calcium sulphate	0.124
Potassium bromide	0.103
Strontium sulphate	0.288
Boric acid	0.282

Took all this salt and dissolved in distilled water to make 1 litre

Bioassay

Ten shrimp were transferred to each sample vial using a 9 in. Disposable pipette and artificial sea water was added to make 5ml. the nauplii can be counted macroscopically in the pipette against a lighted back ground. A drop of dry yeast suspension (3 mg in 5 ml artificial sea water) was added a food to each vial. The vials were maintained under illumination. Survivors were counted, with the aid of a 3x magnifying glass, after 12 and 24 hours and percentage of death at each dose and control were determined

Percentage of death = (control-test/control) x100

IV. Results and Discussion

T.S of the leaf

Transverse section of the leaf shows an isobilateral condition. The upper epidermis single layered with polygonal cells and covered on the outer side by a cuticle. Lower epidermal cells are irregular and thin. The epidermal walls are wavy or sinus while the stomata are paracytic. Trichomes are present. Mesophyll cells are heterogeneous and differentiated into palisade and spongy parenchyma. Upper palisade cells are single layered compactly arranged with elongated narrow, columnar cells and this continues also over the midrib region. Spongy parenchyma is thin, narrow and loosely arranged between the upper and lower palisade. Vascular strands are seen very frequently. Few spharaphides (cluster crystals) are also seen in the parenchyma. Lower palisade is restricted,

unlike upper palisade, to lamina region only. Cells are smaller than those of upper palisade, loosely arranged and their walls are wavy. Chlorophyllus cells are spongy tissue, parenchymatous, 1-3 layered at the lamina portion, multi-layered at the midrib region with polygonal cells, thin walls, achlorophyllous, and with intercellular spaces.

T.S of the midrib presents a flat ventral surface and convex dorsal surface. The epidermal layers are continuous over the midrib. The cells of the lower epidermis however are small with thick cuticle. The cells of the upper palisade are relatively smaller. As mentioned earlier the lower palisade is not represented in the midrib and instead a patch of collenchymas is seen. Vascular bundle is prominent occupying the central portion of the midrib. Xylem as usual is towards the ventral surface and phloem towards the dorsal surface .The vascular bundle is covered on both the sides (dorsal and ventral) by patches of sclerenchymatous fibres.

Fig1: Transverse Section of the Miliusa wayanadica Leaf

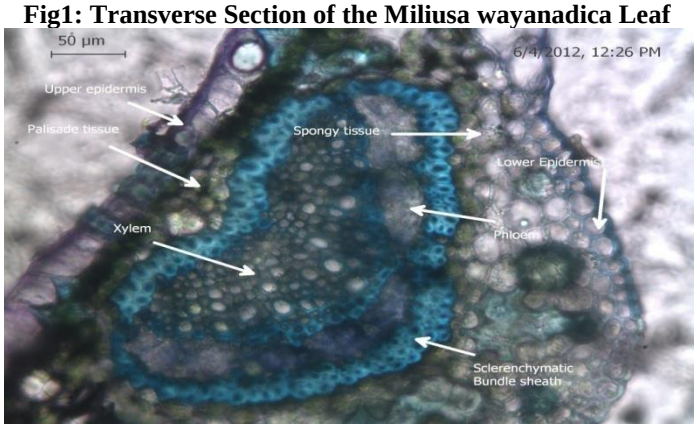


Fig 2: Lower leaf epidermal pealing

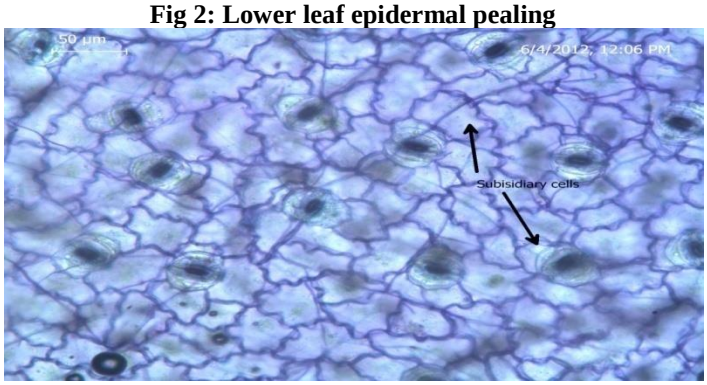


Fig 3: Leaf upper epidermal pealing

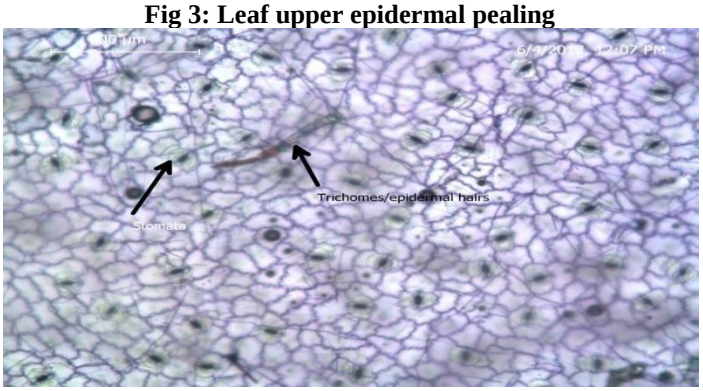
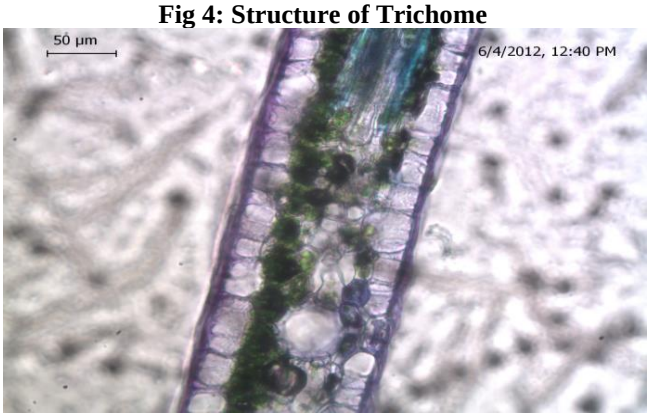


Fig 4: Structure of Trichome



V. Preliminary Phytochemical

Quantitative estimation of phytochemicals in the crude extract shows the presence of alkaloids saponins and flavonoids. These chemicals provide pharmacological properties and also have high antioxidant activity.

Table 1: Preliminary phytochemical screening of ethanolic extract of Miliusa wayanadica

Sl no	Test	Ethanolic Extract
1	Alkaloids	
a)	Mayer's reagent	Present
b)	Dragendroff's reagent	
c)	Hager's reagent	
d)	Wagner's reagent	
2	Saponins	
a)	Foam test	Present
b)	Haemolysis test	
3	Cardiac glycosides	
a)	Legal's test	Absent
4	Flavonoids	
a)	Shinoda test	Present

Fluorescence Analysis

Fluorescence analysis is done to determine the chemical nature of the crude extract. Table represents the fluorescence obtained in short wavelength, long wavelength and daylight.

Table 2: Fluorescence analysis of Leaf extract of Miliusa wayanadica

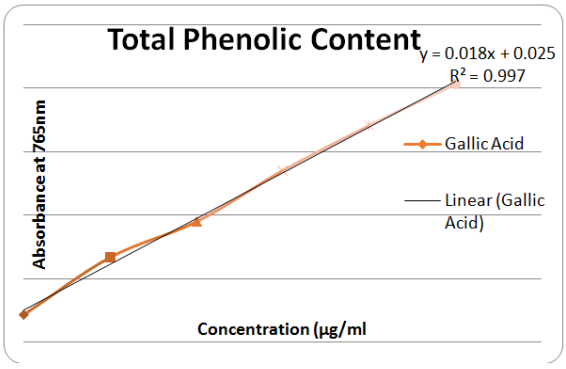
	UV Short wave length	UV Long wave length	Fluorescent
Eth. extract + 50% H_2SO_4	Dark brown	Green	Brown
Eth. extract + 50% HNO_3	Dark green	Light green	Yellow
Eth. extract + Conc. HCl	light violet	Green	Yellow
Eth. extract + Conc. H_2SO_4	Dark violet	Dark green	Reddish brown
Eth. extract + Conc. HNO_3	Purple violet	Greenish yellow	Yellow
Eth. extract + Methanol	Light violet	Light green	Colourless
Eth. extract + Methanolic KOH	Light violet	Light green	Light yellow
Eth. extract + 10% $FeCl_3$	Violet	Green	Orange
Eth. extract + Ammonia	Dark green	Light green	Yellow
Eth. extract + KOH	Light violet	Light green	Light yellow

Eth. extract = Ethanolic extract

Total Phenolic Content

Phenolic compounds are commonly found in both edible and inedible plants, and have been reported to have multiple biological effects, including antioxidant activity. Phenolics are able to scavenge reactive oxygen species due to their electron donating properties. The phenolic compounds such a phenolic acid and flavonoids are most important antioxidant food source. The total phenolic content was determined using Folin- Ciocalteau method, reported as gallic acid equivalents with reference to standard curve Figure ($y = 0.018x + 0.025$ and $r^2 = 0.997$). The total phenolic content extract was calculated to be 15.6% wt/wt GAE/g of extract.

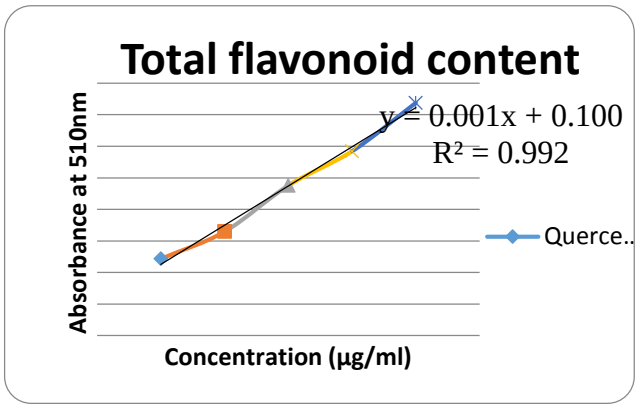
Concentration (µg/ml)	Gallic Acid
0	0.0217
2	0.0674
4	0.0956
6	0.1353
8	0.1707
10	0.2035



Total Flavonoid Content

Total flavonoid contents were measured using aluminium chloride colorimetric assay. Whereby the flavonoids content was 60%wt/wt of quercetin equivalent per gram of sample by reference to standard curve (y = 0.001x + 0.100 and r^2 = 0.992).

concentration (µg/ml)	Quercetin
100	0.2432
200	0.3292
300	0.4761
400	0.584
500	0.7379
100	0.2432

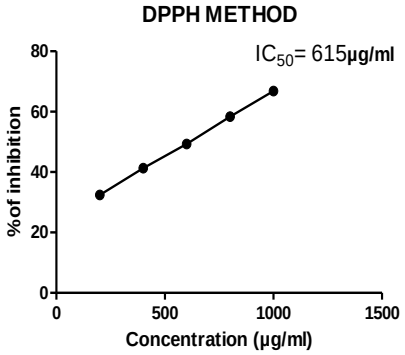


DPPH Method

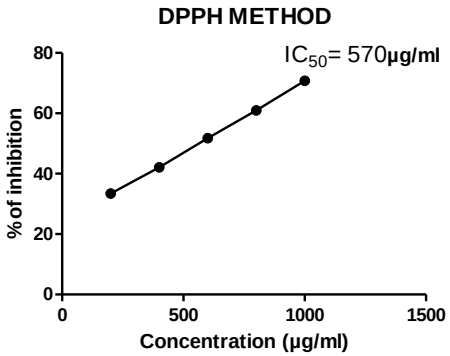
DPPH method measures the ability of antioxidant to react with stable free radical, DPPH and convert it to 1, 1, diphenyl 2-picryl hydrazine. Antioxidant activity is expressed as the concentration required to inhibit 50% free radicals (IC_{50}). The ethanolic plant extract showed an IC_{50} value of 465µg/ml as compared to BHT (570µg /ml) and BHA (615µg/ml) taken as reference. These results show the ethanolic extracts of the plant have exhibited high antioxidant activity. The present study is proof that, leaf extract of Miliusa wayanadica has the potential antioxidant compounds.

Concentration (µg/ml)	**% Radical Scavenging Assay**		
	BHA	**BHT**	**Ethanolic Extract**
200	32.43	33.46	38.12
400	41.3	42.09	46.91
600	49.28	51.57	56.33
800	58.34	60.95	64.9
1000	66.82	70.77	75.28

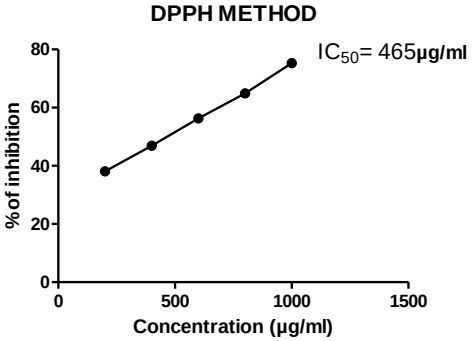
BHA - DPPH Method



BHT – DPPH Method



Ethanolic Extract – DPPH Method

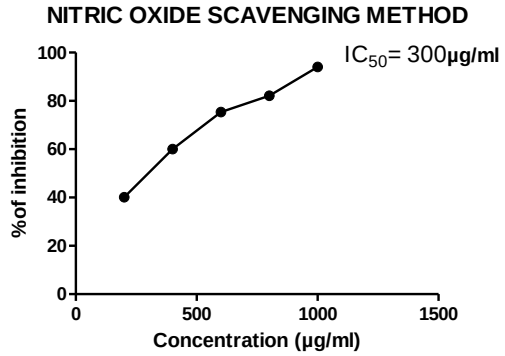


Nitric Oxide Scavenging Activity

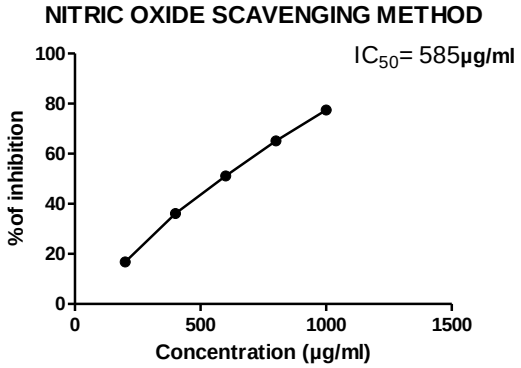
The nitric oxide is a very unstable species under the aerobic O_2 to produce the stable product nitrates and nitrite. It is estimated by using the Griess reagent. The test compound, which acts as the scavenger, reduces the amount of nitrous acid. The extent of decrease reflects the extent of scavenging. Ethanolic extract of Miliusa wayanadica showed an IC_{50} value of 585µg/ml while the standard ascorbic acid had 300µg/ml.

Concentration (µg/ml)	**% Radical Scavenging Assay**	
	Ascorbic acid	**Ethanolic Extract**
200	40.1	16.8
400	60.0	36.1
600	75.4	51.1
800	92.0	65.1
1000	104.4	77.47

Ascorbic Acid – Nitric Oxide Scavenging



Ethanolic Extract-Nitric Oxide Scavenging

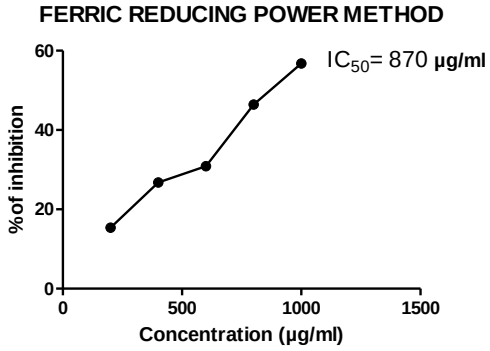


Ferric Reducing Antioxidant Power Method

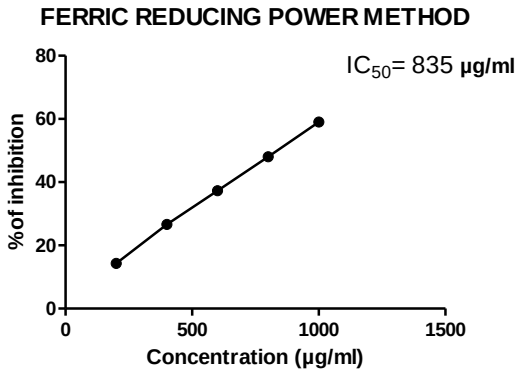
The FRAP test measures the ability of samples to reduce ferric ion to the ferrous form in the presence of TPTZ (2, 4, 6-tripyridylstriazine) to form a blue coloured Fe^{2+}-TPTZ complex. Arbitrarily, one FRAP unit is defined as the reduction of 1 mol of Fe^{3+} to Fe^{2+}. Standards BHT and BHA had IC50 of 835µg/ml and 870µg/ml respectively while ethanolic extract of Milliusa wayanadica shows an IC_{50} of 600µg/ml.

Concentration (µg/ml)	**% Radical Scavenging Assay**		
	BHA	**BHT**	**Ethanolic Extract**
200	15.4	14.3	27.52
400	26.8	26.6	37.60
600	30.9	37.3	50.92
800	46.44	48.0	60
1000	56.76	59.0	73.0

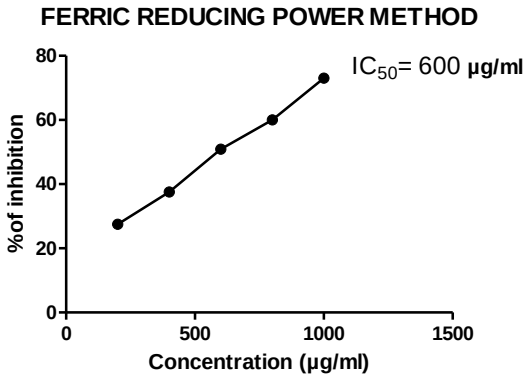
BHA- Ferric Reducing Power Method



BHT – Ferric Reducing Power Method



ETHANOLIC EXTRACT –Ferric Reducing Power Method

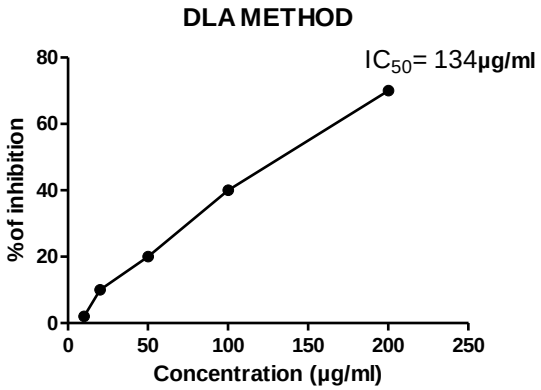


DLA and EAC Method

In vitro cytotoxicity of the ethanolic extract was studied using the Ascetic tumor cells such as Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC). The ethanolic extract showed a dose dependent inhibitory activity against DLA and EAC cells at a dose range of 0-250 µ g / ml. the result shows IC50 of DLA and EAC had 134 µg/ml and 125 µg/ml respectively.

DLA Method

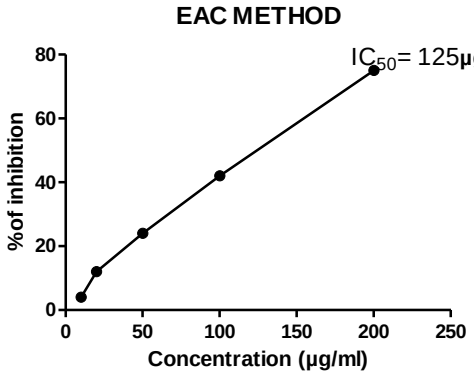
Drug concentration (µg/ml)	**Percent cell death (DLA)**
200	70%
100	40%
50	20%
20	10%
10	2%



Control tube contains only one dead cell.
The sample dissolves in DMSO.

EAC Method

Drug concentration (µg/ml)	Percent cell death (EAC)
200	75%
100	42%
50	24%
20	12%
10	4%



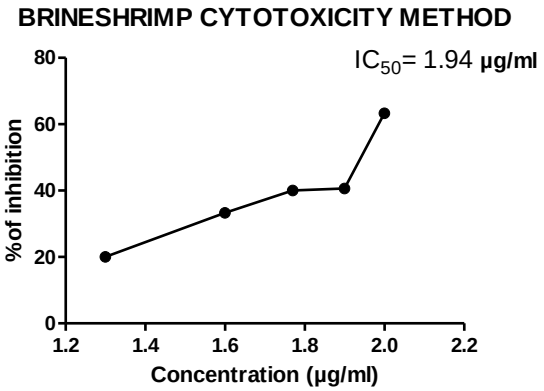
Control tube contains only one dead cell.
The sample dissolves in DMSO.

VI. Effect Of Miliusa wayanadica Leaf Extract On Brine Shrimp Toxicity

The brine shrimp was hatched by using artificial sea water and percentage of mortality by ethanolic extract was calculated while the result shows the IC50 of 1.94 µg/ml.

Sl no	EXTRACT CONCENTRATION (µ gm/ml)	No.of dead organisms (Artemia salina) after 12 hrs.			No.of dead organisms (Artemia salina) after 24 hrs.		
		1	2	3	1	2	3
	20	1	1	2	1	2	3
	40	3	3	4	3	3	4
	60	4	4	4	4	4	4
	80	5	5	4	5	5	4
	100	6	6	7	6	6	7

Log dose(extract) µ/ml	Percentage of mortality
1.3	20%
1.6	33.33%
1.77	40%
1.90	40.66%
2	63.33%



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