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RESEARCH ARTICLE

PHYSICOCHEMICAL CHARACTERISTIC AND COMPARATIVE IN-VITRO ANTI-MICROBIAL ACTIVITY OF LEAVES, STEM, BERRIES OF *Laurus nobilis.L* And Anti OXIDANT ACTIVITY OF LEAVES OF *Laurus nobilis.L* OF A MALAYSIAN SPECIES

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ABSTRACT

Plants are natural sources consists of many natural derived products. In ancient times the herbals are taken in raw to cure much disease, the development of science and technology investigates that there are some active compounds which plays the major role in curing the disease. Various plant and its parts such as leaf, stem, fruit and roots contains the different types of active substances against the inhibition dreadful diseases. In this current study *Laurus nobilis.L* parts were extracted using the suitable solvent and studied for the various types of microbial assay and anti oxidant activity. The stem and fruit extracts shows no inhibition activity where as the leaf extract shows the bioactivity against the *Pseudomonas sp, Escherichia coli* and no effect on *Vibrio cholera* and fungi. The bay leaf extract shows 60.57% activity in xanthine oxidase assay,for DDPH assay 5 mg/ ml s 77.59% activity, cupric shows the 60.83% activity in 5 mg/ ml and Beta Carotene Assay as 69.25% respectively.

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INTRODUCTION

Herbal medicines have been main source of primary healthcare in all over the world. From ancient times, plants have been catering as rich source of effective and safe medicines. About 80 % of world populations are still dependent on traditional medicines. Herbal medicines are finished, labeled medicinal products that contain as active ingredients, aerial or under ground part of plants or other plant materials, or combination thereof, whether in the crude state or as plant preparations. Medicines containing plant materials combined with chemically defined active substances, including chemically isolated constituents of plants are not considered to be herbal medicines (Ajay Kumar Meena *et al.,* 2009). The various role of herbal plants research is shown in the Figure 1.

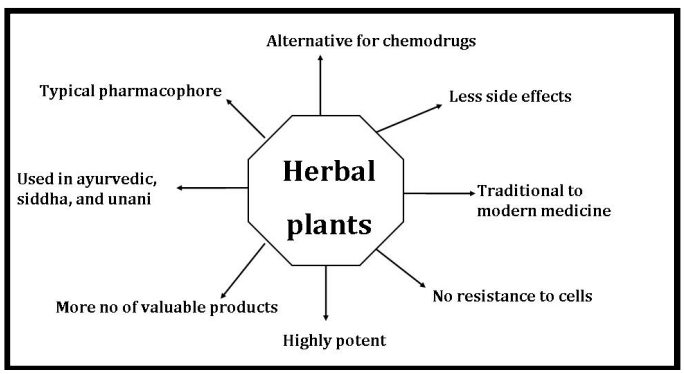

Figure 1. Role of herbal plants

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Drug discovery from medicinal plants has played an important role in the treatment of cancer and, indeed, most new clinical applications of plant secondary metabolites and their derivatives over the last half century have been applied towards combating cancer. (Newman *et al*., 2000, 2003; Butler, 2004) In a natural products drug discovery program, bioassay plays an important role. A bioassay will be applied to large numbers of initial samples to determine whether or not they have any bioactivity of the desired type. It will be used to select materials for detailed individual study. And a bioassay will be used to guide fractionation of a crude material towards isolation of the pure bioactive compounds For these purposes, bioassay tests must be simple, rapid, reliable, reproducible, sensitive, meaningful and, most importantly, predictive. (Suffness, 1987). *Laurus nobilis* is an evergreen Tree growing to 12 m (39ft) by 10 m (32ft) at a slow rate. It is hardy to zone 8. It is in leaf 12-Jan it is in flower from Apr to May. The flowers are dioecious and are pollinated by Bees. The plant is not self-fertile. The most abundant essential oil found in laurel is cineole, also called eucalyptol. The leaves contain about 1.3% essential oils, consisting of 45% eucalyptol, 12% other terpenes, 3-4% sesquiterpenes, 3% methyleugenol, and other α- and β-pinenes, phellandrene, linalool, geraniol, and terpineol. (Vaughan *et al.*, 2009) The bay tree has a long history of folk use in the treatment of many ailments, particularly as an aid to digestion and in the treatment of bronchitis and influenza. (Phillips and Foy, 1990). It as many medicinal properties such a anticancer (Panza, *et al.*, 2011), antioxidant (Ramos, *et al.*, 2012) and anti bacterial (Ramos *et al.*, 2012). In this current study the different parts of *Laurus nobilis* were taken studied for various assay of anti bacterial activity and anti oxidant activity.

MATERIALS AND METHODS

Plant materials

The plant materials of *Laurus nobilis*, were collected from Bujang Valley, Archeological site, Merbok, Kedah, Malaysia. Taxonomic

identification was made from USM, Malaysia (Specimen herbarium no: 11250). A voucher specimen is preserved in our laboratory for further reference at school of Pharmaceutical sciences, University Sains Malaysia.

Preparation of Extracts

The plant material, were dried under shade, segregated, pulverized by a mechanical grinder and passed through a 40 mesh sieve. The powdered plant materials were successively extracted with methanol by hot continuous percolation method in Soxhlet apparatus11 for 24 hrs. The extracts were concentrated by using a rotary evaporator and subjected to freeze drying in a lyophilizer till dry powder was obtained.

Agar diffusion method

The extracts (stem, leaf, and fruit) obtained from the customer, was weighed and dissolved in DMSO to get a final concentration of 1mg/ ml. Anti bacterial activity against Pseudomonas, and Vibrio cholerae, was studied. Each microorganism was inoculated in the Nutrient broth in test tubes, and kept for overnight incubation. After overnight incubation, the microorganisms were flooded on the surface of the pre sterilized Muller Hinton Agar plate. Two wells, each 10mm in diameter, were cut from the Muller Hinton agar plate which has been flooded with the test micro organisms. A 100microliters (1000ppm) of crude extract (test sample) was loaded in to one well and antibiotic (cephalosporin) of same concentration was loaded into the other well. The plates were incubated for 24 hours at 37 ± 3◦C. (Salie *et al.,* 1996 and Newton *et al*., 2002).

Minimal Inhibitory Concentration

The minimal inhibitory concentration (MIC) was determined by both broth dilution assay and agar diffusion assay methods. For broth dilution assay method, a 24 hour pure culture of *E. coli* was grown in Muller Hinton broth. From the broth, 1000µl of culture was taken and inoculated individually into a set of 5 test tubes that already contained 10ml of fresh Muller Hinton broth. 5 different concentrations of plant extract (1ppm, 10ppm, 100ppm, 1000ppm, 2000ppm) were prepared for testing against *E.coli*. One ml volume of each concentration of plant extract was transferred to the tubes accordingly. The contents in the tubes were mixed thoroughly and incubated overnight at room temperature. The MIC end point is the lowest concentration of plant extracts at which there is no visible growth in the form of zone or turbidity is noticed in the tubes. (Langfield *et al.,* 2004).

Minimal Bactericidal Concentration

After MIC determination of the plant extracts an aliquot of 10µl from the inoculated into the fresh Nutrient agar broth. The tubes were kept monitored for culture growth once in one hour for 5 hours by measuring the absorbance at 600 nm using UV-VIS spectrophotometer. Mean while, a 10µl aliquot of plant extracts from tubes which showed no visible growth in MIC test were seeded into Nutrient agar plates and kept for overnight incubation at room temperature. (Taylor *et al*., 1983).

Antifungal Assay

The extracts (stem, leaf, fruit) obtained, was weighed and dissolved in DMSO (1mg in 1ml). Anti fungal activity against *Penicillin sp and Aspergillus sp* was studied. The micro-organisms were inoculated in the Potato dextrose broth in test tubes and incubated for 4 hours at room temperature and humidity. After 4 hours of incubation, the culture was flooded on to the surface of sterilized Potato dextrose agar plate. Two wells, each 10mm in diameter, were cut from the potato dextrose agar plate. A 100 microliters (1000ppm) of crude extract was loaded into one well and antibiotic (Chlorompenicol) of same concentration was loaded in to the other well. The plates were incubated for 3 days at room temperature. All the plates were examined for zones of growth inhibition. (Muyideen *et al.,* 2013)

DPPH Assay

Antioxidant scavenging activity was studied using 1,1-diphenyl-2-picryl hydrazyl free radical (DPPH) (Blois, M. S. 1958)with slight modification. 1.0 ml of test solution was added to 2.0 ml of 0.004% solution of DPPH in methanol. 1ml of Methanol and 2ml of 0.004% DPPH solution was used as experimental control. After 30 min of incubation at room temperature, the reduction in the number of free radical was measured by reading the absorbance at 517 nm. The scavenging activity of the samples corresponded to the intensity of quenching DPPH. The percent inhibition was calculated from the following equation.

% inhibition = [(Absorbance of control – Absorbance of test sample)/Absorbance control] x100.

Cuprac Assay

The cupric ion reducing capacity was measured according to the method of Apak et al. (2006). 1 mL CuCl2 (10 mM), 1 mL neocuproine (7.5 mM), and 1mL NH4Ac buffer (1M, pH 7.0) solutions were added into a test tube. Then, 0.5 mL different concentrations of extract were mixed and total volume was brought up to 4.1 mL with deionized water. The mixture absorbance was recorded against a blank at 450 nm after 30 min incubation at room temperature. The results of the assay were evaluated by using EC_{50} values. (Apak *et al.,* 2006)

% inhibition = [(Absorbance of control – Absorbance of test sample)/Absorbance control] x100.

Xanthine oxidase inhibition assay

The XO oxidase activity was assayed spectrophotometrically under aerobic conditions, based on the procedure reported I by (Noro *et al.,* 1983) The assay mixture ,consists of 50micro litre of test solution,35micro litre of 0.1mM phosphate buffer(PH=7.5),and 30micro litre of enzyme solution(0.01 units/ml in 0.1mM phosphate buffer,pH=7.5) was prepared immediately before use. After preincubation at 25degree centigrade for 15minutes, the reaction was initiated by the addition of 60micro litre of substrate solution (150mM xanthine in the same buffer). The assay mixture was incubated at 25degree centigrade for 30minutes.Absorbance at 290nm was measured with spectrophotometer. A blank was prepared in the same manner. One unit of XO was defined as the amount of enzyme required to produce 1nmol of uric acid/minutes at 25degree centigrade. Xanthine oxidase inhibitory activity is expressed as the percentage inhibition of XO in the above system, calculated as (1-B/A) X 100,where A and B are the activities of the enzyme without and with test material.

Beta Carotene Assay

The beta carotene bleaching method was carried out to measure the antioxidant activity. Beta carotene(0.2mg/ml) dissolved in chloroform, was added to round bottom flasks(50ml) containing 20micro litre of linoleic acid and 200micro liter of Tween 20.A volume of 200micro litre of 80% MeOH(as control) or corresponding plant extract or BHT (as standard) added to the mixture. The concentration of the BHT and the plant extract are same (40mg/ml). After evaporation to dryness under vaccum at room temperature, oxygenated distilled water (50ml) was added and the mixture was shaken to form a liposome solution. The samples were then subjected to thermal auto oxidation at 50degree centigrade for 2hrs.The absorbance of the solution was monitored at 470nm by taking measurements at 10min intervals for 120min and the rate of bleaching of beta carotene was recorded. (Dapkevicius *et al.,* 1998)

RESULTS AND DISCUSSION

Various Microbial assays for in-vitro screening

The methonolic extracts of leaf, stem and fruits of *Laurus noilbis* against microorganism such as *Pseudomonas sp* and *Vibrio cholera* were studies, after 24 hrs of incubation, the results are observed and tabulated in the Table 1 for agar diffusion method. All the plates were examined for zones of growth inhibition and the diameters of these zones were measured in centimeters.

Table 1. Antibacterial activity of different Methanolic extracts

S.No	Methanolic Extracts of	Organisms studied	
		Pseudomonas sp	*Vibrio cholera*
1	Leaf	1.2cm	No activity
2	Stem	No activity	No activity
3	Fruit	No activity	No activity
4.	Cephalosporin	2.3cm	2.1cm

The stem and fruit extracts shows no inhibition activity where as the leaf extract shows the bioactivity against the *Pseudomonas sp* and no effect on *Vibrio cholera.* In this study the antibiotic Cephalosporin was used as a standard. Followed by the MIC end point is calculated for the plant extracts in agar diffusion and broth assay is shown in the Table 2 and Figure 2 for broth assay and Figure 3 for agar diffusion method. At which there is no visible growth in the form of zone or turbidity is noticed in the tubes. No visible growth indicates inhibition of the extract against the microorganism in leaf extract at 1000ppm and 2000ppm.on other hand in this assay the stem extract also shows no visible growth at 2000ppm.The slight growth of microorganism was noticed in the leaf extract at 100ppm and stem extract at 1000ppm. The minimum inhibitory concentration against *E.coli* for leaf extract was found to be 1000 ppm and for stem extract it was found to be 2000 ppm

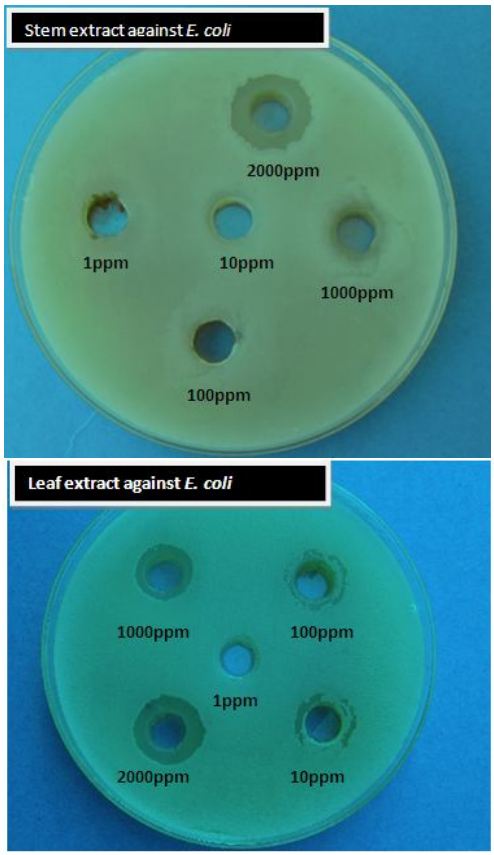

Figure 3. The activity of leaf extract and stem extract against *E.coli* by agar diffusion assay method

Table 2. Broth dilution assay for the three samples for *E.coli*

S. No	1ppm	10ppm	100ppm	1000ppm	2000ppm
Leaf extract	Visible bacterial growth	Visible bacterial growth	Slight growth	No growth	No growth
Stem extract	Visible bacterial growth	Visible bacterial growth	Visible bacterial growth	Slight growth	No growth
Fruit extract	Visible bacterial growth	Visible bacterial growth	Visible bacterial growth	Visible bacterial growth	Visible bacterial growth

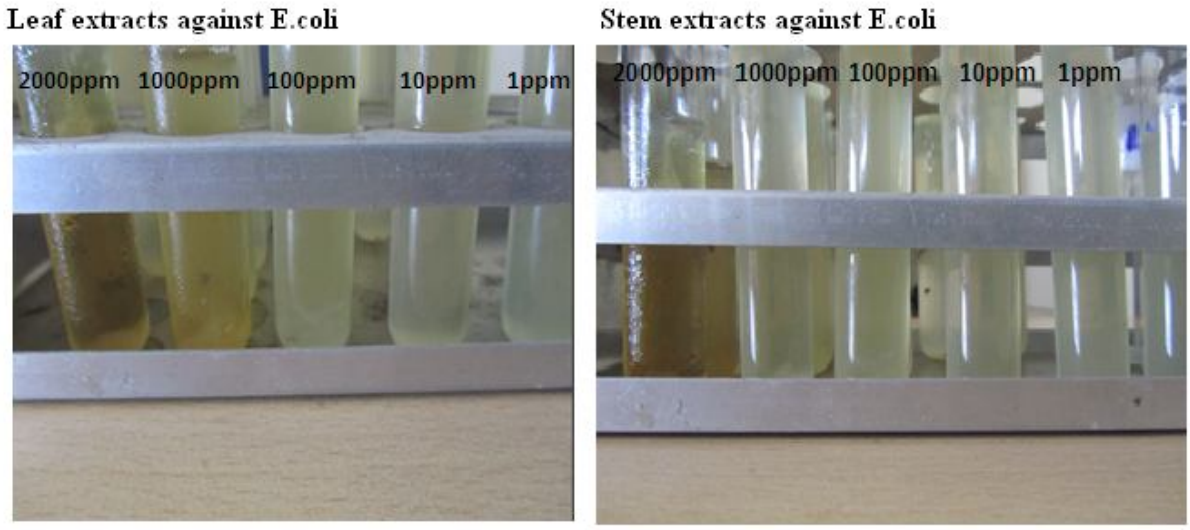

Figure 2. Antibacterial activity of leaf and stem extract against *E.coli* at different concentrations

The MBC endpoint is defined as the lowest concentration of antimicrobial agent that kills >99.9% of the initial bacterial population where no visible growth of the bacteria was observed on the nutrient agar plates is shown in the Table 3 and Figure 5.

The Methanolic Extracts of leaf, stem and fruit shows no more activity against the fungi *Aspergillus sp* and *Penicillin sp* respectively. The antibiotic Chlorompenicol was used a standard for this anti fungal activity is shown in the Table 4.

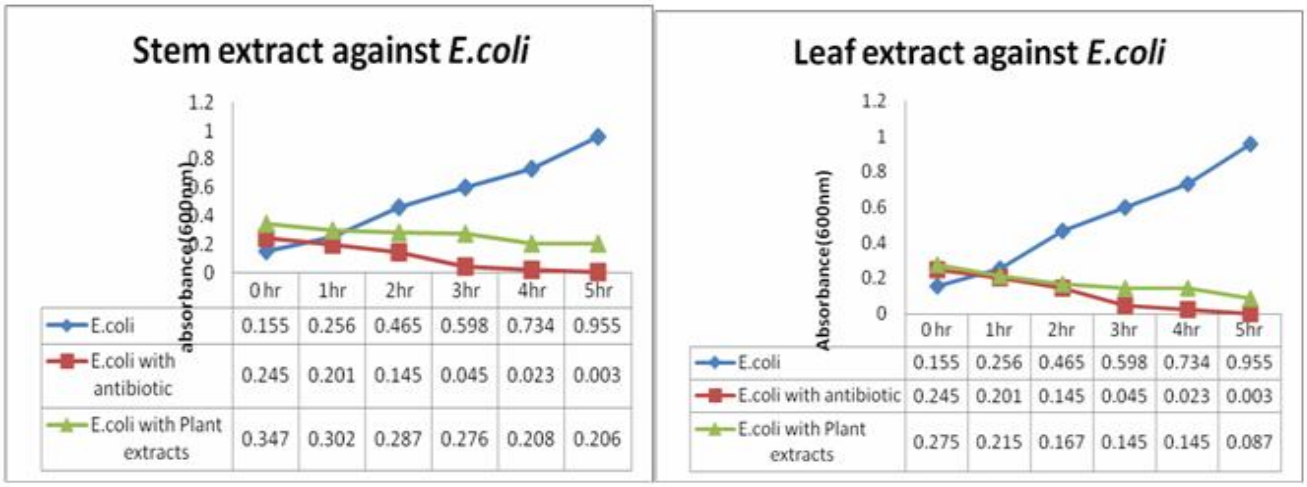

Figure 4. UV-VIS results of minimal bactericidal study of stem extract and leaf extract against *E.coli*

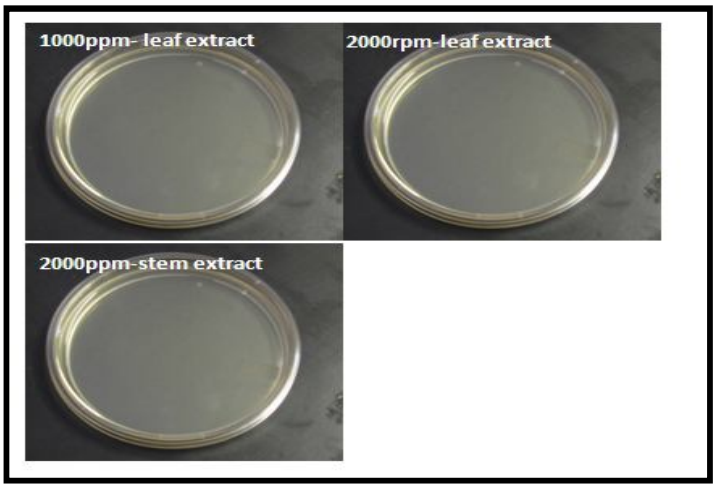

Figure 5. The results of plating(Broth dilution asay)

Table 3. Minimum Bactericidal assay –confirmation by plating(Broth dilution asay)

Sl. no	1000ppm	2000ppm
Leaf extract	No growth found in plates	No growth found in plates
Stem extract	-	No growth found in plates

Table 4. Antifungal activity of different extracts

S.No	Methanolic Extracts of	Organisms studied	
		Aspergillus sp	*Penicillin sp*
1	Leaf	No activity	No activity
2	Stem	No activity	No activity
3	Fruit	No activity	No activity
4.	Chlorompenicol	2.4cm	2.3cm

Table 5. Bay leaf extract-DPPH method

Sample Name	Volume of sample (mg/ ml)	Absorbance	% of activity
Control	-	0.5037	-
Bay leaf extract	0.1	0.4679	7.107
	0.2	0.4122	18.17
	0.3	0.3437	31.76
	0.5	0.2478	50.8
	1	0.1835	63.57
	2	0.1225	75.68
	3	0.1132	77.53
	5	0.1129	77.59
IC50	0.5	-	50.00

Anti-oxidant assay

The extracts are tested for the anti-oxidant activity using different assay and the results are shown in the below Figure 6 and Table 5 for DPPH assay.

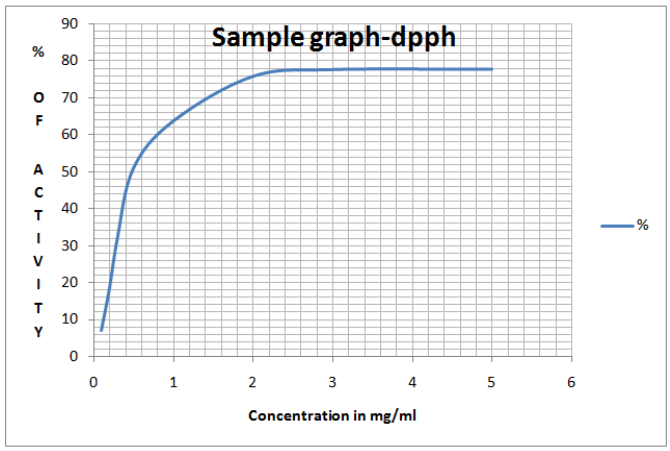

Figure 6. Bay leaf extract-DPPH method

Table 6 and Figure 7 for cupric cuprac assay method. For DDPH assay 5 mg/ ml shows the 77.59% activity and cupric shows the 60.83% activity in 5 mg/ ml.

Table 6. Bay leaf extract- CUPRAC assay method

Sample Name	Volume of sample (mg/ 0.5ml)	Absorbance	% of activity
Control	-	0.6143	-
Bay leaf extract	0.1	0.6040	1.68
	0.5	0.5197	15.40
	1.0	0.4732	22.97
	2.0	0.3337	45.68
	3.0	0.2811	54.24
	4.0	0.2414	60.70
	5.0	0.2406	60.83
EC50	2.4	-	50.00

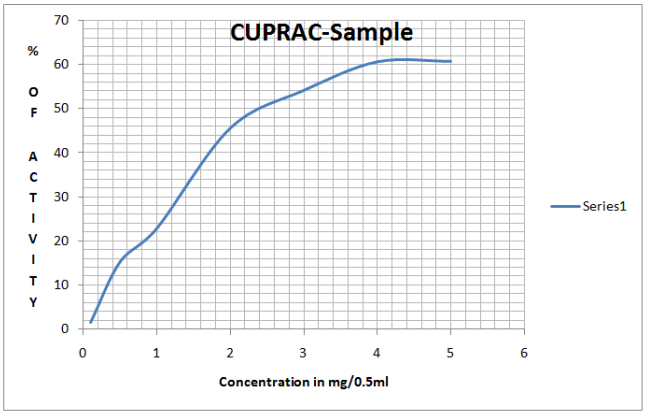

Figure 7. Bay leaf extract- CUPRAC assay method

Similar type of anti-oxidant assay using the leaf bay extract for xanthine oxidase inhibition assay and beta carotene assay is shown in the Table 7 and 8 and Figure 8 and 9 for xanthine oxidase and with Quercitin as a standard for Beta Carotene Assay BHT as a standard. The bay leaf extract shows 60.57% activity in xanthine oxidase assay but the standard Quercitin shows 80% activity at 500ppm. Beta Carotene Assay shows 69.25% activity for bay leaf extract ,standard BHT shows 60% activity at 500ppm

Table 7. Bay leaf extract- xanthine oxidase assay method

Sample	Concentration(ug)	Antioxidant activity %
	500ppm	12.18%
	2500ppm	24.72%
Bay Leaf Extract	5000ppm	33.21%
	10000ppm	60.57%
Quercitin std	500ppm	80%

Table 8. Bay leaf extract- Beta Carotene Assay

Sample	Concentration(ug)	Antioxidant activity %
	500ppm	15.83
	2500ppm	27.74
Bay Leaf Extract	5000ppm	38.42
	10000ppm	69.25
BHT	500ppm	60

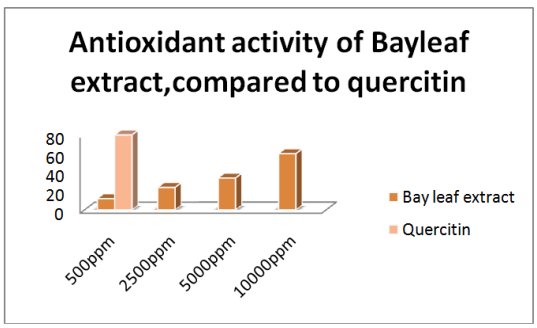

Figure 8. UV-VIS spectrophotometer studies for xanthine oxidase assay

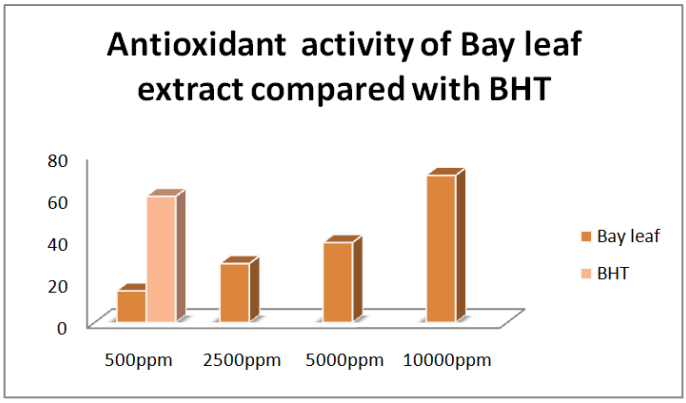

Figure 9. UV-VIS spectrophotometer studies for Beta Carotene Assay

Conclusion

Hence, the *Laurus noilbis* shows the promising microbial activity and anti-oxidant activity. thus further analytical studies such as HPLC, column chromatography and NMR are need to be carried to find the pure active ingredient for maximum activity of inhibition, so that in future it can be used a alternative for the chemodrugs, and the side effects due to the chemical drugs can be minimized.

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