Determine of the antioxidant and antimicrobial activity of *Azadirachta Indica* extract

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Abstract

The result obtained was that the methanolic extract of *Azadirachta indica,* neem leaves have some phytochemical compounds in it. It consists of chemicals like diterpenes nimbiol, triterpenes: β- sitosterol, stigmasterol (leaf). Number of cyclic tri and tetrasulphide (leaves), Flavonoids: nimaton, quercetin, myrcetin, kaemferol. By performing the antioxidant experiments with DPPH, NO scavenging free radicals activity, H2O2 activity tests shows much scavenging property in the methanol extract rather than pet ether and chloroform leaf extract. Whereas in the Phenolic content it is showing the presence of phenol content is more in methanol extract than the PLE and CLE. So, by this result basis we concluded that the antimicrobial activity in determination of MIC should be performed with MLE because this contains more phenol in it and has been undergone with successive extraction procedure with the three solvents according to its polarity and so it concludes the antimicrobial activity and antioxidant activity was more.

Key Words: *Azadirachta indica,* antioxidant and antimicrobial activity.

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1. Introduction

NATURE always stands as a golden mark to exemplify the outstanding phenomenon of symbiosis. The biotic and abiotic elements of nature are all interdependent. The plants are indispensable to man for his life. The three important necessities of life – food, clothing and shelter- and a host of other useful products are supplied to him by the plant kingdom. Nature has provided a complete store house of remedies to cure all ailments of mankind. The knowledge of drugs has accumulated over thousands of years as a result of man's inquisitive nature so that today we possess many effective means of ensuring health-care. The history of herbal medicines is as old as human cultivation[1] .In the 19th century, the term 'Materia Medica' was used for the

subject now known as "Pharmacognosy". Pharmacognosy may be defined as a branch of bioscience which treats in detail medicinal and related products of crude or primary type obtained from plants, animal and mineral origins. Pharmacognosy is an impor5tant link between Pharmacology and Medicinal chemistry. As a result of rapid development of phytochemistry and pharmacological testing methods in recent years, new plant drugs are finding their way into medicine as purified phytochemical, rather than in the form of traditional galenical preparations. Though several countries in the world have a rich heritage of herbal drugs, very few can put claim for their procurement only from cultivated species [2]. It is recently that some of these drugs have been subjected to systemic cultivation based on modern scientific information. Moreover, cultivation of medicinal plants offer wide spectrum of advantages over their wild sources, it may be an uneconomical proposition for certain types of crude drugs which occur abundantly in their natural habitat e.g. nux-vomica, acacia, myrobalan, etc. On the other hand , crude drugs like cardamom, clove, Indian hemp, poppy latex, tea, cinchona, ginger, linseed, isabgol, Ceylon-cinnamon, saffron, peppermint etc are obtained from agricultural plants. The cultivation of vegetable drugs involves convergence of various factors from agricultural and pharmaceuticals sphere, such as soil, climate, rainfall, irrigation, altitude, temperature, use of fertilizers and pesticides, genetic manipulation and biochemical aspects of natural drugs. When all such factors are precisely applied, the new approach to scientific cultivation technology emerges out. Our main objective was to determine the antioxidant and antimicrobial activity from the extract of *Azadirachta indica* as neem leaves[3].

2. Material and Methods

2.1. Methods:

Collection:

Azadirachta indica, as neem fresh and matured leaves were harvested from the Institute of Bengal School of Technology, Hooghly, and Kolkata. Specimens of these leaves were authenticated in Botany Department of in Botanical Garden of West Bengal and voucher specimens deposited in the herbarium. The authenticated number is CNH/Tech.II/2014/83/183.The harvested leaves were selected to remove dead ones and unwanted materials. The leaves were thoroughly washed with tap water to remove all dust particles. The leaves were dried under shed until completely dried and were pulverized into fine powder. The powder was used for extraction method. The extraction was done by using the soxolet apparatus. This method was done by using three solvent according to its polar solvent and it was successive. The three solvents used here was Petroleum Ether, Chloroform and Methanol. This method was carried out for 20days and the temperature was controlled between 15- 20ºC for 7hours every day. After completion of extraction by each solvent, the solvent was evaporated by the distillation apparatus. The extraction was stored in dried and cleaned petridish and kept in the refrigerator at 5ºC [4].

Determination of total ash value:

Take a empty crucible. Weigh the empty crucible in which the experiment to be performed. Powder drug was placed in crucible and proper weigh was taken and note down. Then the crucible containing the drug was slowly carbonized using a muffle furnace at 650ºC. After that when temperature rise to the required degree then for half an hour the temperature was maintained in the furnace. After that the drug content was turned into white ash colour. The weight of the crucible was being noted again and the percentage of the total ash content was calculated.

Procedure for acid soluble ash value:

0.1N of 25ml of dilute hydrochloric acid was added to half of the amount of ash was taken in a glass beaker and heated on a water bath for 10min. Then another glass beaker was rinsed with 5ml of hot water. After that the weight of a filter paper was taken. The rinsing was added to a glass beaker and filtered using a filter paper. The residue was transferred to the filter paper with water and dried and acid insoluble was weighed. Finally the percentage of acid insoluble ash was calculated.

Procedure for water soluble ash value:

25ml of distilled water was taken in a glass beaker and heated it for 10min in a water bath. Then the ash are mixed with warm water. Then filter it in a same way. Water insoluble portion which are present in filter paper was dried and weight and percentage was calculated.

Phytochemical tests

The phytochemical investigation of a plant may involve authentication and extraction of the plant material; separation and isolation of the constituents of pharmaceutical interest; characterization of the isolated compounds; investigation of the biosynthetic pathways to particular compounds; and the quantitative evaluations. The test solution depending upon the type of natural drug under examination , the test solution may be a aqueous extract or alcoholic extract or extract in specific menstrum like Petroleum Ether, Chloroform, Methanol for the tests to be performed. For testing, as far as possible clear, transparent solution should be used [5].

Alkaloid test:

To the extract, dilute hydrochloric acid was added, shaken well and filtered. With the filtrate, the following tests were performed. Dragendraff's reagent: To 2ml of filtrate, add few drops of dragendraff's reagent. The precipitate observed was reddish brown in colour which indicates the presence of alkaloid. Mayer's test: To 3ml of filtrate, few drops of Mayer's reagent were added along sides of tube. Formation of reddish brown precipitate indicates the presence of alkaloids. By dissolving a mixture of mercuric chloride 1.36g, potassium iodide 5g in water 100ml. Most alkaloids are precipitated from neutral or slightly acidic solution by mayer's reagent (potassium iodide solution) to give a cream colour precipitate. Wagner's test: To 2ml of filtrate, few drops of wagner's reagent added in a test tube. Formation of reddish brown precipitate indicates the presence of alkaloids. An aqueous solution of iodine and potassium iodide used for microchemical analysis of alkaloids. Hager's reagent: To 2ml of filtrate, few drops of Hager's reagent were added in a test tube. Formation of yellow color precipitate indicates the presence of alkaloids [6].

Tests for Carbohydrates:

Molisch test: 2ml of aqueous extract was treated with 2 drops of alcoholic α-naphthol solution in a test tube and then 1ml of concentration sulphuric acid was added carefully along the sides of the test tube. Formation of violet ring at the junction indicates the presence of carbohydrates.

Test for reducing sugars: [7]

Fehling's test: To 1ml of aqueous extract, 1ml of Fehling's A and 1ml of Fehling's B solutions were added in a test tube and heated on a water bath for 10minutes. Formation of red precipitate indicates the presence of reducing sugar.

Benedict's test: Equal volume of Benedict's reagent and extract were mixed in a test tube and heated on a water bath for 5-10minutes. Solution appears green, yellow or red depending on the amount of reducing sugar present in the test solution which indicates the presence of reducing sugar.

Test for Flavonoid:[6]

Alkaline reagent test : The extract was treated with few drops of sodium hydroxide solution separately in a test tube. Formation of intense yellow color, which becomes colorless on addition of few drops of dilute acid indicates the presence of flavonoids.

Lead Acetate test: The extract was treated with few drops of lead acetate solution. Formation of yellow precipitate indicates the presence of flavonoids.

Test for Glycosides:[8]

Borntrager's test: To 3ml of test solution, dilute sulphuric acid was added, boiled for 5minutes and filtered. To the cold filtrate, equal volume of benzene or chloroform was added and it was shaken well. The organic solvent layer was separated and ammonia was added to it. Formation of pink to red color in ammonical layer indicates the presence of anthraquinone glycosides.

Legal's test: 1ml of test solution was dissolved in pyridine. 1ml of sodium nitroprusside was added and made alkaline using 10% sodium hydroxide solution. Formation of pink to blood red color indicates the presence of cardiac glycosides.

Keller-Killiani test: To 2ml of test solution, 3ml of glacial acetic acid and 1drop of 5% ferric chloride were added in a test tube. Carefully 0.5ml of concentrated sulphuric acid was added by the sides of the test tube. Formation of blue color in the acetic acid layer indicates the presence of cardiac glycosides [9].

Test for Tannin and Phenolic compounds:

Ferric chloride test: A small amount of extract was dissolved in distilled water. To this solution 2ml of 5% ferric chloride solution was added. Formation of blue, green or violet color indicates presence of phenolic compounds.

Dilute iodine solution test: To 2-3 ml of extract, few drops of dilute iodine solution were added. Formation of transient red color indicates the presence of phenolic compounds[10].

Test for Saponin:

Froth test: The extract was diluted with distilled water and shaken in a graduated cylinder for 15minutes. The formation of layer of foam indicates the presence of saponin.

Test for triterpenoids and steroids:

Salkowski's test: The extract was treated with chloroform and filtered. The filtrate was added with few drops of concentrated sulphuric acid, shaken and allowed to stand. If the lower layer turns red, sterol is present. Presence of golden yellow layer at the bottom indicates the presence of triterpenes.

Libermann- Burchard's test: The extract was treated with chloroform. To this solution few drops of acetic anhydride were added, boiled and cooked. Concetrated sulphuric acid was added through the sides of the test tube. Formation of brown ring at the junction of two layers, if upper layer turns green, indicates the presence of steroids and formation of deep red color indicates the presence of triterpenoids.

ANTI-OXIDANT TESTS [7],[8]

(a) DPPH radical scavenging assay:

A 0.135mm DPPH solution and various concentrations (10-1500µg/ml) of extract were prepared in methanol. 1ml of DPPH solution and 1ml of various test solution were incubated in dark at 25ºC for 20minutes after a gentle vortex. The absorbance was observed at 517nm. The control reaction was carried out without the extract. A graph concentration vs. percentage inhibition was prepared and IC50 value was calculated. The percentage inhibition was calculated by following formula-

% inhibition= [(A0-A1)/A0]*100

[A0= Absorbance of control, A1= Absorbance of test sample]

(b) To determine phenol content in Extract:

Total phenolic were determined using Folin-ciocalten reagent (FCR). Briefly, 100µl of the extract dissolved in methanol (1mg/ml) was mixed with 750µl of FCR (diluted 10-fold) and allowed to stand at 22ºC for 5minutes; 750µl of sodium carbonate (60g/l) solution was then added to the mixture. After 90minutes the absorbance was measured at 765nm. Results were expressed as Gallic acid equivalents.

(c) To determine the total NO content in extract:

In this method nitric oxide generated from sodium nitroprusside was measured by the Greiss reaction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated by using Greiss reagent Scavenger of nitric oxide compete with oxygen leading to reduced production of nitric oxide. Sodium nitroprusside (5mM) in phosphate buffered saline (PBS) was mixed with 3.0ml of different concentration (50, 100, 150, 200, 250, 300µg/ml) pf the extract dissolved in the suitable solvent systems and incubated at 25ºC for 150min. The samples from the above were reacted with Greiss reagent (1% sulphonamide, 2% H3PO4 and 0.1% napthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during the diazotization of nitrite with sulphonamide and subsequent coupling with napthylethylenediamine was read at 546nm and referred to the absorbance of standard solution of potassium nitrite, treated in the same way with Greiss reagent. Ascorbic acid was used as a reference compound. The percentage inhibition was calculated as

% NO radical Scavenging activity= [(Acontrol – Atest)/ Acontrol]*100

(d) To determine flavonoids content in extract:

Total soluble flavonoid content of the fractions was determined with aluminium nitrate using quercetin as the standard. One mg of the fraction was added to 1ml of 80% ethanol. An aliquot of 0.5ml was added to test tubes containing 0.1ml of 10% aluminium nitrate 0.1ml of 1M potassium acetate and 4.3ml of 80% ethanol. The absorbance of the supernatant was measured at 415nm after incubation at room temperature for 40min. The total flavonoid content in the fractions was determined as µg Quercetin equivalent by using the standard quercetin graph. The flavonoid contents of the extracts in terms of quercetin equivalent using the following linear equation based on the calibration curves

A=0.0067, C=0.0132, R^2 =0.999. A is the absorbance, and C is quercetin equivalents (µg). The test was performed in triplicate and average value was represented.

(e) To determine hydroxyl radical (OH^-) scavenging activity:

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and methanol extract of Diospyros peregrina fruits for hydroxyl radical generated by Fe^{3+} - Ascorbate – EDTA – H2O2 system. The reaction mixture contained in a final volume of 1.0ml. 100µl of 28mM 2-deoxy-2-ribose in 20mM KH2PO4-KOH buffer of pH 7.4, 500µl of the selected concentrations of extract (50,100,150,200,250,300µg/ml) in KH2PO4-KOH buffer (20mM, pH 7.4), 100µl of 1.04mM EDTA, 100ml 200mM FeCL3 100µl of 1.0 mM H2O2 and 100µl of 1.0mM ascorbic acid was incubated at 37ºC for 1hour. 1.0ml of thiobarbituric acid (1%) and 1.0ml of trichloroacetic acid(2.8%) were added to the test tubes and were incubated at 100ºC for 20min. After cooling, absorbance was measured at 532nm against control containing deoxyribose and buffer. Ascorbic acid was used as a positive control. Reactions were carried out in triplicate. The percentage inhibition was determined by comparing the results of the test and control compounds. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose degradation and is calculated as:

$$\%OH^- \text{ radical scavenging activity} = [(A_{control} - A_{test})/A_{control}]*100$$

Where Acontrol is the absorbance of the control reaction and Atest is the absorbance in the presence of the sample of the extract. The antioxidant activity of the extract was expressed as IC50. The IC50 value was defined as the concentration (in µg/ml) of extracts that inhibits the formation of OH- radicals by 50% [10].

ANTIMICROBIAL ACTIVITY[9]

The MIC (Minimal Inhibitory Concentration) of a bacteria to a certain antimicrobial agent gives a quantitative estimate of the susceptibility.

MIC is defined as the lowest concentration of antimicrobial agent required to inhibit growth of the organism. The principle is simple: Agar plates, tubes microlitre trays with two-fold dilutions of antibiotics are inoculated with a standardized inoculums of the bacteria and incubated under standardized conditions following NCCLS guidelines. The next day, the MIC is recorded as the lowest concentration of antimicrobial agent with no visible growth.

The MIC informs us about the degree of resistance and might give you important information about the resistance mechanism and the resistance genes involved. MIC-determination performed as agar dilution is regarded as the gold standard for susceptibility testing.

MIC determination by broth dilution in aseptic condition:

2.2. Materials:

Equipment

- ✓ McFarland standard 0.5
- ✓ Disposal loops (1ml and 10ml)
- ✓ Burner

Media

- ✓ Sterile normal saline
- ✓ 50ml cation adjusted Mueller-Hinton II broth
- ✓ Mueller-Hinton agar plates for purity control of inoculum suspension.

Bacterial Strains

- ✓ Salmonella typhi B111, S.typhi A2467, S.typhi C-145, S.typhi E-3404, S.typhi NCTC-74
- ✓ S. aureus ML-15, S. aureus ML-351, S.aureus MM-366, S.aureus ML-276, S.aureus ML-145

Sterilize these media in the autoclave at 120ºC at 15 p.s.i and distribute the MHB in the bijou bottle for organism inoculation and MHA in the McCartney bottle for plate. After inoculation in the MHB media keep the bijou bottle in the incubator for 24hrs. Then add 1ml of drug solution of different concentration(0,1,2.5,5,10,25,50 µg/ml) and mixed it with McCartney bottle containing sterile molten MHA media and drug plate were prepared in 45mm petridish. Plates were placed in freeze overnight for diffusion of drug uniformly in whole plate. For preliminary screening different species of gram positive and gram negative were taken. Before inoculating the organisms directly into the agar plate dilute it with saline water with respect to McFarland standard 0.5 by adjusting the turbidity of inoculums to match that of standard(McFarland 0.5 ˜ approximately 10^8CFU/ml. Standardisation of inoculums is essential because the interpretation of the result is based on a certain inoculums. All the microorganism were inoculated in all drug plates contain different concentration of extract. Plates were then incubated in 37ºC for overnight. The presence and absence of microbial growth on the plates were observed.

3. RESULT:

Table-1 QUALITATIVE PHYTOCHEMICAL ANALYSIS OF THE PETROLEUM ETHER, CHLOROFORM AND METHANOLIC EXTRACT OF AZADIRACHTA INDICA (neem leaves)

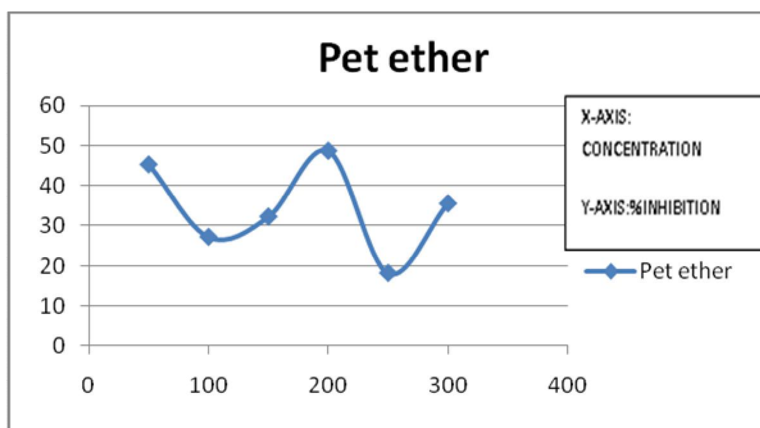
The result of qualitative phytochemical analysis of pet.ether leaf extract(PLE), chloroform leaf extract(CLE) and methanolic leaf extract(MLE) are given in the table below. Results indicate the presence of many phytochemical components in both the extracts.

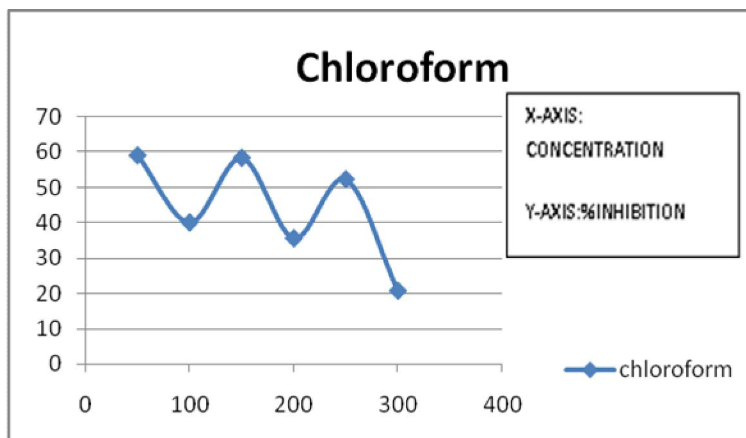
SL. NO.	CONSTITUENT	TEST	PLE	CLE	MLE
1	Alkaloids	Mayer's reagent test	+	+	+
		Wagner's reagent test	+	+	+
		Hager's reagent test	+	+	+
2	Carbohydrates	Molisch's test	+	+	+
3	Reducing sugars	Fehling's test	+	+	+
4	Flavonoids	Alkaline reagent test	+	+	+
5	Glycoside	Legal's test	-	-	+
		Borntrager's test	-	-	+
		Killer- Killiani test	+	+	+
6	Tannins and Phenolic compounds	Ferric chloride test	+	+	+
		Dilute iodine solution test	+	+	+
7	Saponins	Froth test	+	-	+

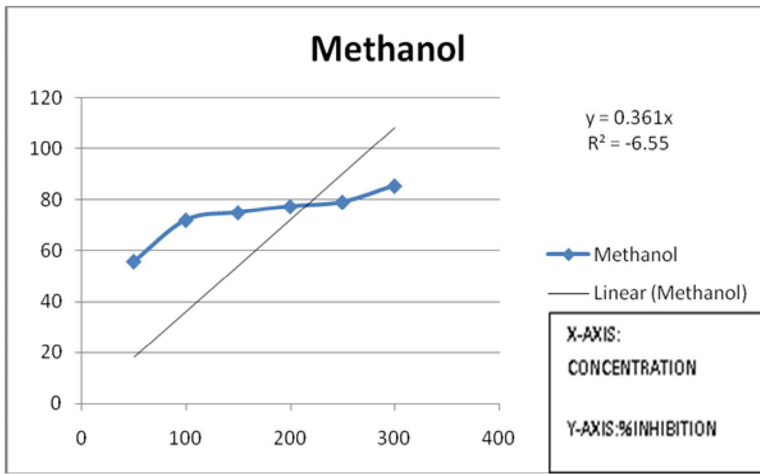
8	Triterpenoids and Steroids	Salwonski test	-	-	+
		Libermann and Burchard's test	-	+	+

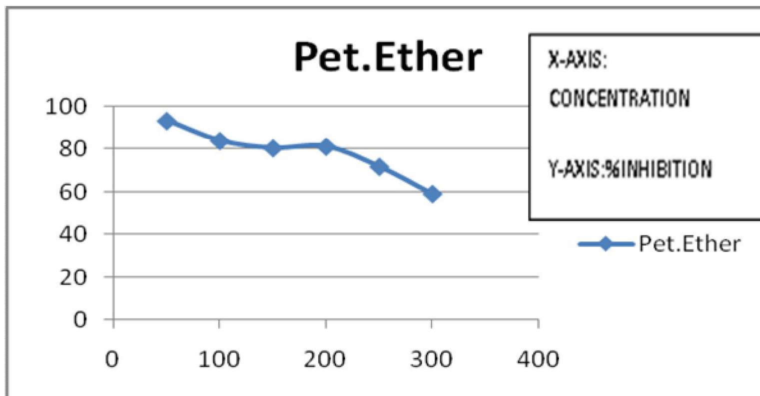
ANTIOXIDANT ACTIVITY:

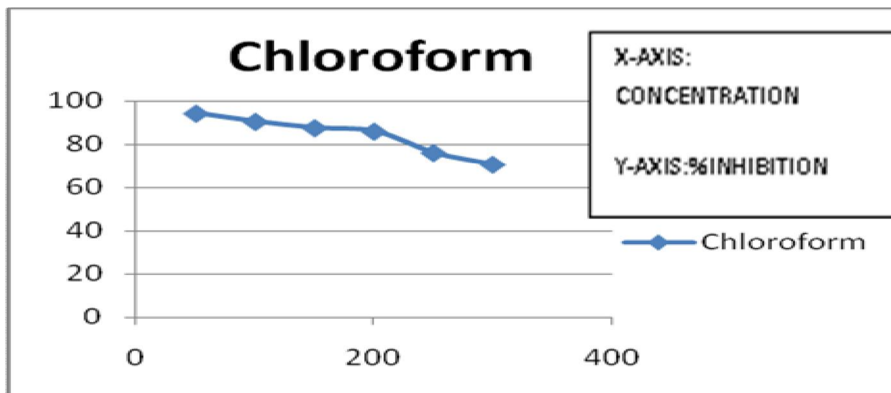
FOR DPPH

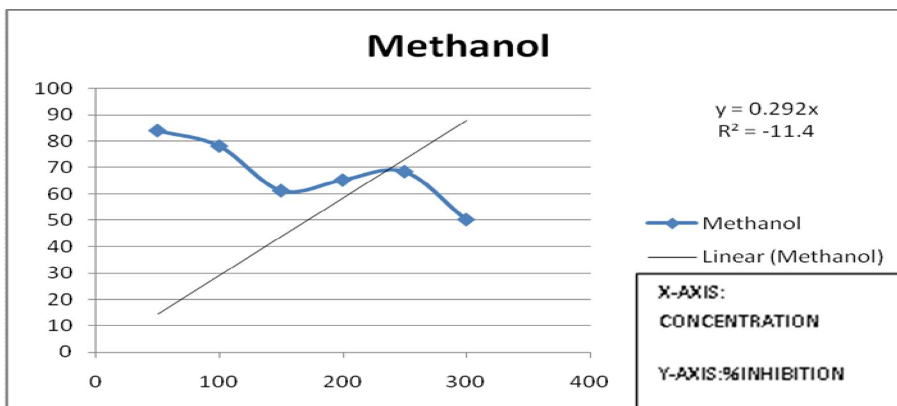




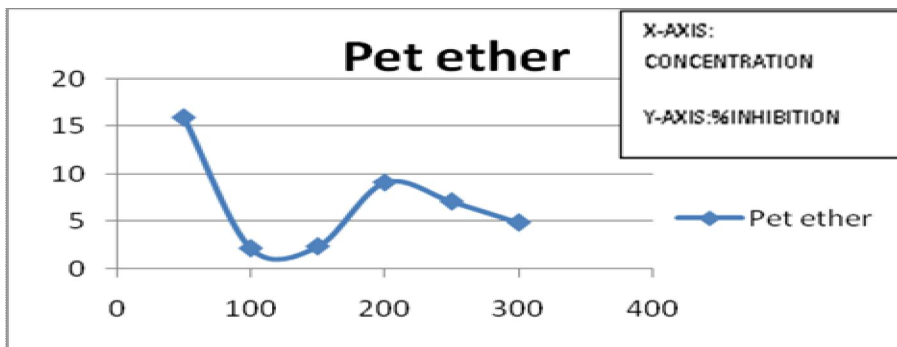


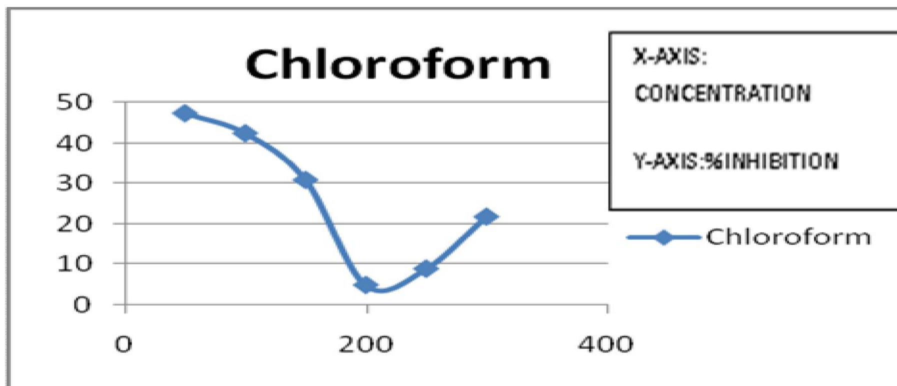






HYDROGEN PEROXIDE CONTENT





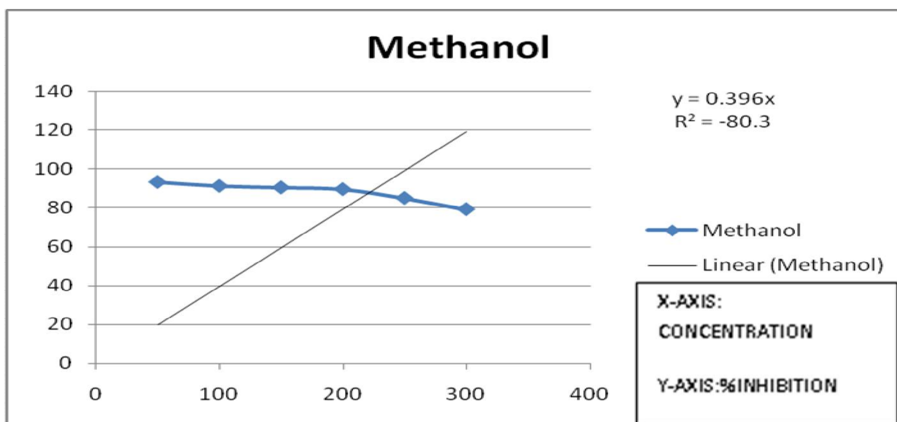


Table 2. ANTIMICROBIAL ACTIVITY:

Conc.(mg) / Organism	1	2.5	5	10	25	50
S.typhi B111	+	+	-	-	-	-
S.typhi A2467	+	+	-	-	-	-
S.typhi C-145	+	+	-	-	-	-
S.typhi C3404	+	-	-	-	-	-
S.typhi NCTC74	+	-	-	-	-	-

S.aureus ML351	+	+	+	+	+	+
S.aureus MM366	+	+	+	+	-	-
S.aureus ML276	+	+	+	-	-	-
S.aureus ML145	+	+	+	-	-	-
S.aureus ML15	+	+	+	+	-	-

Growth = (+)

NO Growth=(-)

4. DISCUSSION:

From this work, I would like to discuss that from the result obtained was that the methanolic extract of *Azadirachta indica,* neem leaves have some phytochemical compounds in it. It consists of chemicals like diterpenes nimbiol, triterpenes: β- sitosterol, stigmasterol (leaf). Number of cyclic tri and tetrasulphide (leaves), Flavonoids: nimaton, quercetin, myrcetin, kaemferol. By performing the antioxidant experiments with DPPH, NO scavenging free radicals activity, H2O2 activity tests shows much scavenging property in the methanol extract rather than pet ether and chloroform leaf extract. Whereas in the Phenolic content it is showing the presence of phenol content is more in methanol extract than the PLE and CLE. So, by this result basis we concluded that the antimicrobial activity in determination of MIC should be performed with MLE because this contains more phenol in it and has been undergone with successive extraction procedure with the three solvents according to its polarity and so it concludes the antimicrobial activity and antioxidant activity was more. So for further studies the Methanolic leaf extract will be taken for the several tests and research work.

5. CONCLUSION:

Methanolic extract of 'NEEM' shows more potency than ether and chloroform. As found in literature survey and experiment we found it's a good antioxidant and antimicrobial agent. Though there are still many spaces for research on this plant to optimize many other potency.

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