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ISOLATION AND PURIFICATION OF NIMBIDIN FROM *AZADIRACHTA INDICA* OF WESTERN GHATS AND EVALUATE ITS ANTIOXIDANT, ANTI-BACTERIAL AND ANTI-DIABETIC PROPERTIES

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Keywords:

Azadirachta indica, Nimbidin, Phytochemical analysis, Antibacterial, Anti-diabetic and Antioxidant activities.

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ABSTRACT: *Azadirachta Indica* has always been known to be the bestowed with good health for many centuries in India. It has been found that the Neem is native to Asian countries. There are three major active components in *A. indica,* namely nimbin, nimbidin, and nimbiene. The compound nimbidin was isolated by chromatographic techniques. *A. indica* leaves extract was prepared, and the phytochemical analysis was conducted, and the phytochemical screening manifested the presence of alkaloids, flavonoids, reducing sugar, phenol, saponins and cardiac glycoside in the neem extract. Then qualitative phytochemical analysis was conducted for flavonoids, alkaloids, saponins, and phenol. Plant sources are considered the major active source of antioxidant molecules for a long time in the history of humankind. In the current study, the anti-bacterial properties of Neem have been tested against gram-positive bacteria, namely, *Staphylococcus aureus*, *Bacillus subtilis,* and gram-negative bacteria, namely, *Escherichia coli Pseudomonas aeruginosa*, *Klebsiella pneumoniae,* and *proteus vulgaris*. Chloramphenicol was used as a standard to analyze the effect of the nimbidin compound. The compounds obtained from plants are mostly categorized by their antioxidant activity. DPPH free radical scavenging activity and Nitric oxide scavenging activity has been performed in our study to determine antioxidant activity of *A. indica*. Diabetes results from an unusual metabolism of insulin. *Azadirachta indica* plays an important role in management of Type II Diabetic Mellitus by improvising the insulin action and secretion.

INTRODUCTION: *Azadirachta indica* is often known as Neem, nim tree, or Indian Lilac, which belongs to the mahogany family in Meliaceae Neem, a very familiar tree with enormous medicinal characteristics.

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Neem is an all-purpose medicinal plant having a wide variety of biological activities. The history of *Azadirachta indica* is inevitably connected to the lineage of the Indian Civilization [1].

Modern research has uncovered the secret efficacy of Neem that the neem leaf has a strong anti-septic, anti-fungal, anti-bacterial, anti-oxidant, anti-inflammatory, anti-carcinogenic, anti-diabetic, anti-parasitic, anti-snake venom anti-HIV, anti-bone resorption, anti-spasmodic, anti-pyretic, anti-diarrheal, anti-helmintic and immuno-modulation properties [2].

Azadirachta indica is a deciduous tree which is commonly found in all parts of the world like India, Africa and South and Central parts of America [3]. All parts of the neem tree have unique medicinal properties. Neem serves to be the most obvious solution for the greater concerns faced by mankind in our global environment. *A. indica* does not have any harmful impact on humans, birds, animals, earthworms and favourable insects. The US Environmental Protection Agency has approved Neem to use on food crops [4].

Neem contains certain chemical components that can help in reducing sugar levels, and it heals ulcers, kills pathogens, etc. Few chemical researches were conducted by Indian pharmaceutical chemist in A. Indica, whereby they extracted an acidic principle in neem oil, which they termed as “margosic acid”. However, the real chemical research was originated later after few decades with isolation of three active components, *i.e*., nimbin, nimbidin, and nimbiene. Nimbidin is a major crude bitter principle that is extracted from the oil of seed kernels of *Azadirachta Indica*. It is demonstrated for several biological activities. Neem is considered to be a precious source of natural products for drug development. The US National Academy of Sciences had acknowledged the significant usage of Neem. There was a report issued in 1992 which was entitled ‘Neem - a tree for solving global problems [5].

Natural bioactive compounds are found in different parts of the plant, *i.e.*, fruit, flower, stem, leaf, root. It provides definite physiological action on the human body. It is widely used in human therapy, veterinary, agriculture, scientific research, and countless other fields. Medicinal plants are utilized for discovering and screening phytochemical compounds, which are helpful in the production of new drugs.

A tree that is loaded with many numbers of compounds that can be used for medicinal purposes and the tree is *Azadirachta indica* [6]. The phytochemical analysis defines the separation, screening, and identification of the bioactive compounds found in plants. The biologically active components that can be acquired from plants are flavonoids, alkaloids, carotenoids, phenolic, antioxidants and tannin compounds.

Chemically constituents may be therapeutically active or inactive. Several phytochemical researches have been carried out for detecting various groups of naturally available phyto-chemicals. Previous investigations on *A. indica* have manifested that it consists of active compounds with a variety of medicinal uses. In traditional medicine, *Azadirachta indica* has been used for the treatment of diabetes. Since neem leaves have antimicrobial properties and it could be used for controlling airborne bacterial contamination in residential communities [7]. The antimicrobial effectiveness of essence of *A. indica* leaves, seed, bark, and roots exhibit high, moderate, and low activities [8]. The pathogenic bacterial infections can be treated with nano-emulsion of neem leaves at lower proportions as an effective anti-bacterial agent without any harm to human systems [9]. The purpose of this study is to isolate the compound Nimbidin from *Azadirachta indica* of Western Ghats to investigate its antioxidant, anti-bacterial and anti-diabetic properties.

MATERIALS AND METHODS:

Collection of Raw Material and Solvent Extraction: Healthy and uninfected Neem leaves were collected. The leaves were washed under running tap water to eliminate dust and other foreign particles. The fresh leaves were crushed, ground, and mixed in 1:10 ratio with ethanol. The extractions were obtained through continuous grinding using mortar and pestle, which was further filtered by using Whatman No.1 filter paper. Then the filtrates were stored at 4 °C.

Chromatographic Methods to Purification Nimbidin: The separation of Nimbidin compound from Neem extracts can be isolated by Thin Layer Chromatography. The glass slides were cleaned and dried in a hot air oven. The preparation of slurry was involved by mixing silica gel and a little quantity of calcium sulfate with distilled water. One blob of the prepared slurry was placed on a slide to make a thin film. The resultant slides were activated by heating in a hot air oven at 110 °C for 30 min.

Loading of Sample: The filtrate obtained using filtration was loaded at the center of the slide 2 cm above the edge. The development tank was saturated with ethanol and water in the ratio of

80:20. The slides were kept in the tank without touching the baseline by the solvent. The final solvent front was marked, and the slides were dried.

Visualization of Compound: For visualization of Nimbidin was viewed under 560 nm UV light. Alkaloids were visualized under UV light, and they were visible as yellow and orange fluorescent spots.

Confirmation of Compound: High-Performance Liquid Chromatography is quite an organized approach for the segregation of substances. It is a well-suited method for analysis of a vast range of application fields.

HPLC is a separation technique for the compounds of an organic mixture of components when those components are non-volatile, unstable compounds and having comparatively high molecular weights.

Phytochemical Analysis: The phytochemical study was performed on the extracts by using the below standard tests.

Test for Alkaloids: To 2 ml of plant extract, few drops of Wagner's solution were added. The appearance of Brown (or) Reddish precipitate show the presence of alkaloids.

Test for Flavonoids: To 2 ml of plant extract, 1 ml of 2N NaOH was added. The presence of yellow color determines the presence of flavonoids.

Test for Cardiac Glycosides: To 2 ml of plant extract, few drops of Con H_2So_4 were added. The formation of red color indicates the presence of Cardiac glycosides.

Test for Amino acids: To 2 ml of plant extract, 2-5 drops of Ninhydrin solution were added. The samples were kept in a boiling water bath for 1-2 minutes. The appearance of purple color indicates the presence of amino acids.

Test for Tannins: With 2 ml of plant extract, 2 drops of 5% ferric chloride solution were added. The presence of tannin was indicated by yellow color.

Test for Reducing Sugar: 2 ml of plant extract was taken in a test tube and 2 ml of Fehling's solution was added. The sample was kept in a water bath for 40 ℃. The formation of red brick precipitation shows the presence of reducing sugar.

Test for Steroids: To 1 ml of plant extract, 2 ml of acetic anhydride and 3-5 drops of chloroform were added. To the test tube, 2 drops of con.H_2SO_4 were added. The appearance of blue or green color indicate the presence of steroids.

Test for Phenols: 2 ml of plant extract was taken in a test tube, and few drops of 10% aqueous Ferric Chloride solution was added. The formation of blue or green color depicts the presence of phenol.

Test for Saponins: To 2 ml of plant extract, 2 ml of distilled water was added, and it was shaken vigorously. The presence of foam on top of the sample indicates the presence of saponins.

Test for Anthraquinones: 2 ml of plant extract was taken in a test tube, and few drops of 10% NH_3 solution were added. The formation of pink color indicates the presence of anthraquinone.

Anti-bacterial Activity: Anti-bacterial capability of Nimbidin was tested against gram-positive bacteria, namely, *Staphylococcus aureus*, *Bacillus subtilis*, and gram-negative bacteria, namely, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *proteus vulgaris*.

The cultures of the test bacteria were kept on nutrient agar slants. The subcultures of the above bacterial species were prepared by inoculating the mother culture of test bacteria aseptically into the clear nutrient broth. The inoculated broth test tubes were incubated overnight at 37 °C.

The cultures were then used to evaluate their sensitivity to the leaf extract by agar well diffusion. Muller-Hinton agar was used to perform the anti-bacterial assay. The cultures were inoculated by swabbing in the sterile nutrient agar plates. By using a cork borer, the wells were made in the agar plates for about an 8 mm diameter. Then the wells were labeled and filled with 25 µl, 50 µl, 75 µl and 100 µl of extract in the wells respectively (25 mg/ml of DMSO). Chloramphenicol was used as the reference antibiotic. The plates were kept undisturbed for half an hour and were incubated at 37 °C for 24 h. The zones of inhibition were measured.

Antioxidant Activity:

DPPH Radical Scavenging Activity: Using 2, 2-diphenyl-1-picrylhydrazyl (DPPH), The atom scavenging activity of methanolic extract of plant extract is often measured. As per the procedure, the scavenging activity for DPPH free radicals was measured. A small portion of 0.5 to 2.5 µl of plant extract/ascorbic acid and an aliquant of 3 ml of 0.004% DPPH solution in methanol was mixed at different concentrations.

The mixture was agitated and let to achieve a gradual state at temperature for thirty min. Using 0.1 cc of various vehicles rather than plant extract/ascorbic acid, a control was produced. The inhibition percentage of DPPH radicals of the plant extract or compound can be deduced by contrasting the values of absorbance of the control and the experimental tubes.

$$\text{Scavenging activity \%} = A_{518}\text{ (control)} - A_{518}\text{ (sample)} \times 100/ A_{518}\text{ (control)}$$

Nitric Oxide (NO) Scavenging Activity: The NO scavenging activity of the sample was deduced by the addition of of 400 µL of 100 mM SNP (sodium nitroprusside), 100 µL of PBS and 100 µL of various concentration of extract [10]. The mixture was kept undisturbed for incubation at 25 °C for 150 min. 0.5 ml of the reaction mixture was taken and 0.5 ml of Griess chemical agent was additional there to (0.1 mL of sulfanilic acid and 200 µL naphthylethylenediamine dichloride ((0.1%)w/v). It was incubated at room temperature for 30 minutes then absorbance was computed at 540 nm. All the reactions were performed and their inhibition percentage at 37° C was calculated by the subsequent formula:

$$\text{Scavenging activity \%} = A_{540}\text{ (control)} - A_{540}\text{ (sample)} \times 100/ A_{540}\text{ (control)}$$

Anti-diabetic Activity:

Alpha-Amylase Activity: The starch iodine method was used to carry out Alpha-amylase activity. 0.01 ml of α-amylase solution (0.025 mg/mL) was added to 0.39 ml of phosphate buffer (0.02 Molarity containing 0.006 Molarity NaCl, pH 7.0) consisting various concentration of extracts. After incubation at 37 °C for 10 min, 0.1 ml of starch solution (1%) and 5 ml of distilled water were added to the above solution.

Then the mixture was further incubated for about one h. After incubation, the absorbance was measured at 565 nm using a UV spectrophotometer. Enzyme Inhibition activity was calculated by the following formula.

% Inhibition = Absorbance of Control - Absorbance of Sample × 100/ Absorbance of Control

RESULTS:

TLC Analysis: There are so many components present in Neem, and the components can be isolated through various methods such as filtration, extraction, distillation, chromatography, and so on. This is one of the most important methods for characterization and identification of the components present in *A. indica*. The compound Nimbidin was visualized under 560 nm Ultra Violet light. It was visible as pink fluorescent spots. The Nimbidin compound visualization is represented in **Fig. 1**.

FIG. 1: VISUALIZATION OF TLC PLATE UNDER UV TRANSILLUMINATOR

HPLC Analysis: 10 µL of the sample was inoculated into the HPLC by using an auto-injector. The segregation of Nimbidin was attained using acetone I trile-water in the ratio 40:60 (1000 µL min-1). The peaks are detected at the wavelength of 214 nm. Online degasser was used to do online degassing with helium.

The variation of the peak of the compound Nimbidin is shown in **Fig. 2**. The compound nimbidin was eluted at a retention time of 10.165 and 11.403 min at areas 6.472% and 5.068%, respectively. The height of the peak of the compound at 10.165 and 11.403 min were 9.796% and 4.3% shown in **Table 1**. The commercial nimbidin detected its peak value at 11.20 min given in **Table 2**.

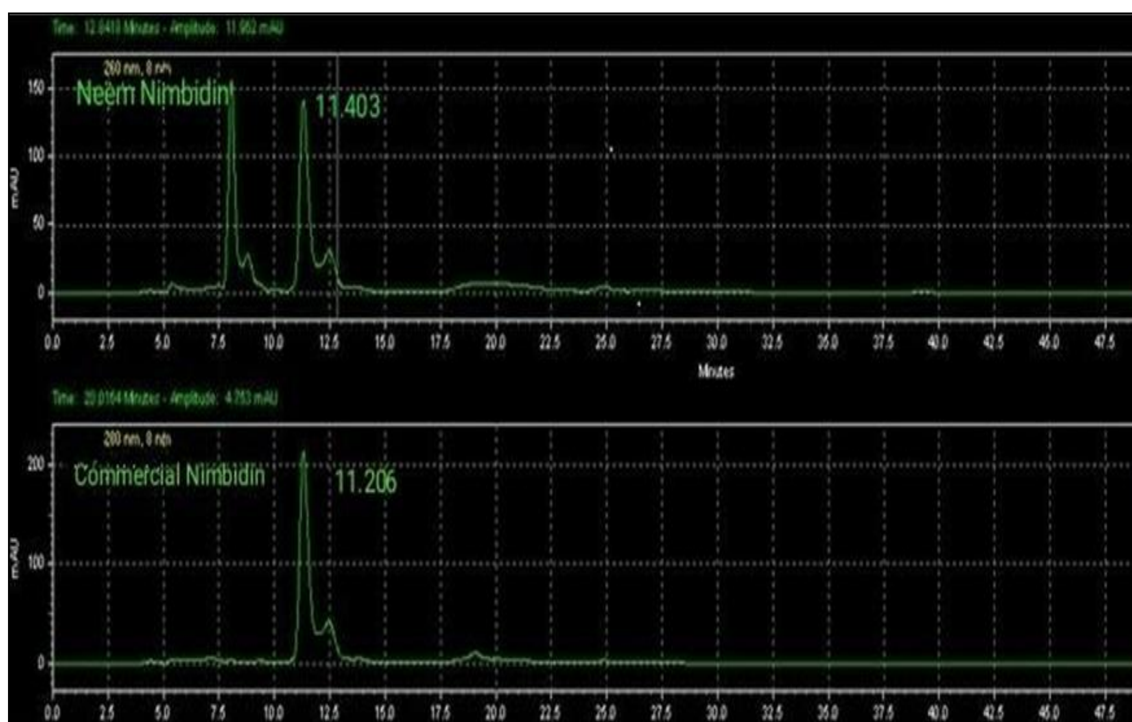

FIG. 2: HPLC CHROMATOGRAM

TABLE 1: NIMBIDIN FROM NEEM EXTRACT

Peak	Retention Time	Area (%)	Height (%)
1	10.165	6.472	9.796
2	11.403	5.068	4.300

TABLE 2: COMMERCIAL NIMBIDIN

Peak	Retention Time	Area (%)	Height (%)
1	11.20	5.028	4.374

Preliminary Phytochemical Analysis: The Phytochemical screening test of extract of *Azadirachta indica* leaves exhibited the presence of different specialized metabolites. The secondary metabolites prominent in the phytochemical screening are Alkaloids, flavonoids, reducing sugars, phenols, saponins. The resultant of the preliminary phytochemical analysis is presented in **Table 3**.

Anti-bacterial Activity: By scrutinizing the cultured agar plates, the anti-bacterial activity of leaf extract of *A. indica* was tested against both gram-positive and gram-negative bacterial species. Leaf extract of *A. indica* showed an added inhibition zone against *Pseudomonas aeruginosa*, *proteus vulgaris*, *Staphylococcus aureus,* and *Klebsiella pneumonia*, whereas the susceptibleness of *Escherichia coli* and *Bacillus subtilis* to the Neem leaves extract were low, which are shown in **Fig. 3**. Diameters of the zone of inhibitions are given in **Table 4**.

TABLE 3: PHYTOCHEMICAL ANALYSIS

Phytochemicals	Plant Extract
Alkaloids	+++
Flavonoids	+++
Cardiac Glycosides	+
Amino Acids	-
Tannins	-
Reducing Sugar	+++
Steroids	-
Phenols	++
Saponins	++
Anthraquinone	-

TABLE 4: ZONES OF INHIBITION

Test Organism	Zone of Inhibition (diameter in mm)				
	Standard	25 µL	50 µL	75 µL	100 µL
Pseudomonas aeruginosa	200	185	197	200	210
proteus vulgaris	300	230	250	300	310
Staphylococcus aureus	190	140	170	190	200
Klebsiella pneumonia	200	170	175	190	250
Escherichia coli	190	40	90	110	170
Bacillus subtilis	160	30	100	145	150

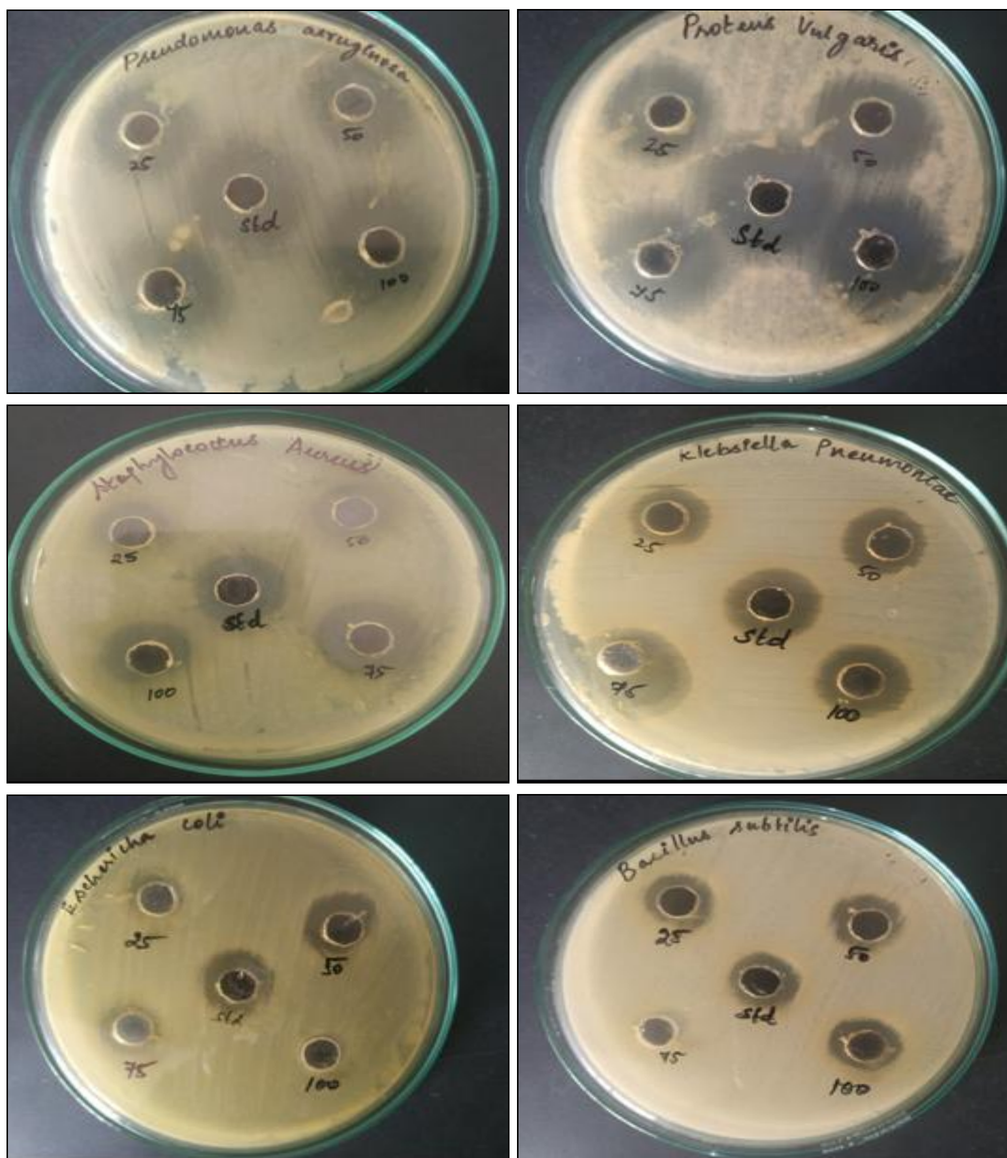

FIG. 3: AGAR PLATES WITH ZONES OF INHIBITION

Antioxidant Activity:

DPPH Scavenging Activity: The results of the DPPH scavenging activity of *Azadirachta indica* are shown in **Fig. 5**. The expanse of the free radical scavenging at various concentrations (10-50 µl) from the extract of Neem was computed, with ascorbic acid as standard. The scavenging activity of the *A. indica* extract was relatively lower than the standard. The resultant of the DPPH scavenging activity exhibited that 50 µl plant extract revealed the maximum inhibition activity of 70.06 %, proceeded by a reduction in inhibition activity in a lower concentration.

Nitric Oxide (NO) Scavenging Activity: The results of Nitric Oxide scavenging activity of *Azadirachta indica* is shown in **Fig. 6**. The extent of Nitric Oxide scavenging at various concentrations (10-50 µl) from the extract of Neem was estimated, with ascorbic acid as standard. The scavenging activity of the *A. indica* extract was relatively lower than the standard. The result of the NO scavenging activity exhibited that 50 µl plant extract revealed the maximum inhibition activity of 66.66 %, proceeded by a reduction in inhibition activity in a lower concentration.

Anti-diabetic Activity:

Alpha-Amylase Activity: The outcome of Alpha-Amylase activity of Neem is shown in **Fig. 7**. The absorbance of the *A. indica* extract at various concentrations were measured. The Alpha-Amylase activity of neem extract was relatively lower than the acarbose.

The result of Alpha-Amylase activity exhibited that 50 µl plant extract revealed the maximum inhibition activity of 75.01% proceeded by the reduction in inhibition activity in a lower concentration.

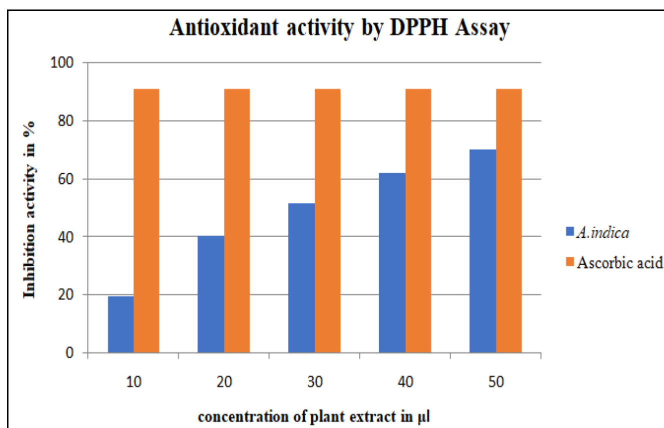

FIG. 4: ANTIOXIDANT ACTIVITY BY DPPH ASSAY

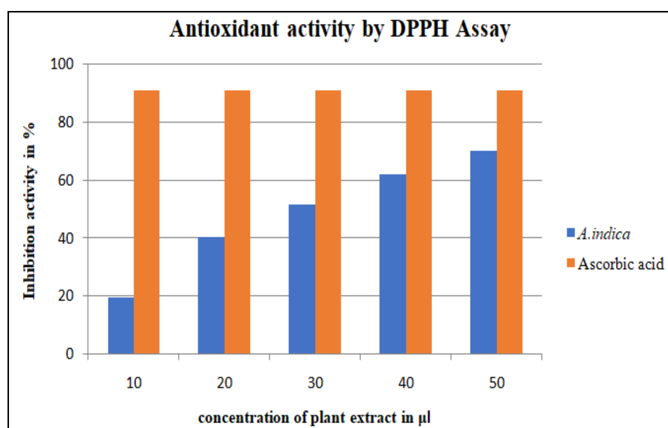

FIG. 5: NO SCAVENGING ACTIVITY

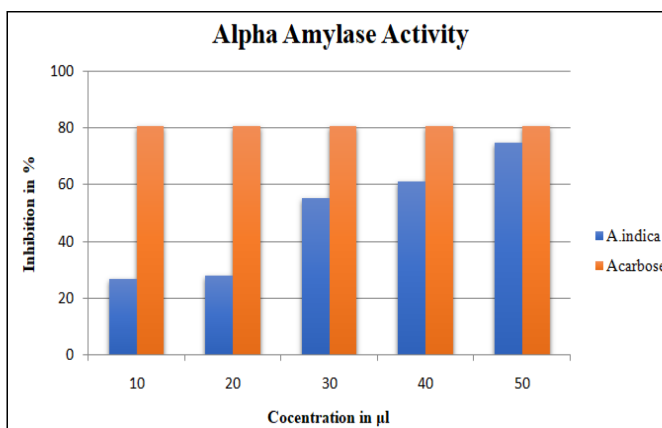

FIG. 6: ALPHA-AMYLASE ACTIVITY

DISCUSSION: *Azadirachta indica* extract have been used for a more years than we can imagine in food preservation, medicine, and in many habitual therapies. Neem leaves extracts are the possible source of antimicrobial compounds, most specifically against bacterial species. Kubmarawa *et al.,* 2008 [10] reported that Nimbidin was one of the compounds that has antimicrobial, antioxidant, and anti-diabetic activities. *In-vitro* studies of anti-microbial activity exhibited that *A. indica* leaves extract inhibited the growth of the bacterial species on different effectiveness levels. The pharmaceutical merits of the secondary metabolites are because of substances that create a physiological operation in humans. The most predominant secondary metabolites are alkaloids, steroids, flavonoids, resins, fatty acids, tannins, and gums for the growth of cells, repairing cells, and bodybuilding.

Singh V and Chauhan D 2014 [11] reported that alkaloids, glycosides, and flavonoids have many biological effects anti-diabetic, anti-viral, anti-cancer, antioxidant, anti-leprosy, anti-allergic, anti-inflammatory, and antimicrobial activities, *etc*. Few diseases like ulcers, malaria, diarrhea, swollen liver, dysentery, *etc*., can be cured by phyto-chemicals. The different kinds of phytoconstituents obtained from several herbs and herbal extracts have biological actions that can be of a worthy therapeutic index non-nutrient compounds like phytochemicals have a protective effect on medicinal plants. Pokhrel Bharat *et al.,* 2015 [12] has discussed that the crude extract of *Azadirachta indica* consists of biochemical and phytochemical active compounds like nimbin and nimbidin azadirachtin, salanin, etc. which can be efficacious against multidrug-resistant pathogens. They also serve as a major provenance of antioxidant.

Pillai NR and Santhakumari G 1981 [13] have observed a major significant effect on lowering blood sugar levels in rabbits treated with nimbidin

orally. Girish K and Bhatt S 2008 [14] reported a biologically active compound, Nimbidin, that can be extracted from *A. indica* seed kernels, leaves, and various parts of the tree. It was discovered that the compound posses different medicinal characteristics such as hypoglycemic, antiulcer, spermicidal, antifungal, anti-inflammatory, antiarthritic, antipyretic, anti-bacterial, anti-diabetic, and antioxidant properties. The inhibition made by the extract of the plant against the microorganism relies on different external and internal parameters.

Cristiane P 2009 [15] described that the extracts procured from different chromatography through various solvents were analogous. The perceptibility of chromatography depictions for every extraction method and the solvent utilized allowed to process the qualitative and quantitative alteration in secondary metabolites. HPLC had been reported by Uebel *et al.,* 1979 [16]; Schneider. B and H and Ermel K 1986 [17] in the literature for the segregation and isolation of the compounds present in *A. indica*. Stokes. JB, and Redfern. RE 1982 [18] and Barnby *et al.,* 1989 [19] reported that few researchers had used HPLC techniques to monitor the residues of compounds after its revelation to sunlight and Ultra Violet radiations.

Jessinta *et al.,* 2013 [20] described that neem oil also possesses antimicrobial properties similar to Neem leaves extract. The resultant of the experiment of neem oil tested against *E. coli*, P. *aeruginosa*, *B. subtilis,* and *R. meliloti* for antimicrobial activity caused the maximum zone of inhibition against *E. coli* and minimum zone of inhibition against *P. aeroginosa*. Most of the bacterial species showed the more or less same size of inhibition zone at various concentrations. Although, the effect of inhibition of the neem oil escalated with a surge in the concentration for every test microorganisms. The most effective zone of inhibition was produced by ciprofloxacin. Parashar. G *et al.,* 2018 [21] have reported that using a combination of two leaf extracts of Lantana camara and a high concentration of *Azadirachta indica* on strains of bacteria shows a higher zone of inhibition. It manifests drastic antibacterial effect on microbes like *E. coli*, *P. aureginosa*, *S. aureus* and *B. subtilis*. Karimi *et al.,* 2010 [22] reported some potential antioxidant compounds present in Azadirachta indica plant, which was confirmed by the *in-vitro* antioxidant activity test. The antioxidant activity was calculated in terms of percentage inhibition of DPPH free radical. Prochazkova *et al.,* 2011 [23] discovered that percentage inhibition was more when a higher concentration of plant extract was used. By using a high concentration of plant, extract depicts that there may be a high amount of antioxidant involved. The presence of flavonoid was indicated by a phytochemical screening of plant extract. It might be responsible for the antioxidant activity. Blois MS 1958 [24] reported that as a steady free radical, DPPH is often used method to detect radical scavenging activity of bioactive compounds. In the radical form of DPPH, It absorb 517 nm, but on reducing the antioxidant activity, the absorption reduces due to the development of its non-radical form. The most significant chemical mediator fabricated by neutrons, endothelial cells, macrophages, *etc*., is Nitric Oxide (NO). Lata H and Ahuja GK *et al.,* 2003 [25] accounted for Nitric Oxide also indulging in balancing various physiological and psychological processes. Ialenti *et al.,* 1993 [26] reported that excessive concentration of Nitric Oxide is associate with few diseases. Ross R 1993 [27] described that the generation of Nitric Oxide take place in biological tissues by a process called nitric oxide synthesis (NOSS). In which converts Arginine to Citralline with the production of nitric oxide through a five electron oxidative reaction.

Frier BM and Fisher M 2006 [28] reported the insufficiency of insulin impacts the metabolism of proteins, fats, carbohydrates and it plays an important role in the disorder of water and electrolyte equilibrium. Both α-amylase and α-glucosidase are significant in glucose absorption and carbohydrate digestion. The inhibitor of α-glucosidase decelerates the digestion of carbohydrates and slackens the absorption.

Satyanarayana K *et al.*, 2015 [29] reported that by feeding an effective oral dose of neem leaves extract for 30 days to the high-fat-diet-induced diabetes rats, the fasting blood glucose, serum lipid profile, levels of insuin signaling molecules and oral glucose tolerance, glucose and glycogen oxidation in gastrocnemius muscle was determined. Bijauliya RK *et al.*, 2018 [30] have interpreted that by improvising the insulin signaling molecules and

glucose utilization in skeletal muscle, neem leaf extract plays a major role in the control of T2D – Diabetes mellitus type 2.

Davis SN and Granner DK 2001 [31] reported Miglitol and Acarbose are competitive inhibitors of and α-glucosidase and slow down starch and disaccharides absorption. The Inhibitors of alpha-amylase behave as an anti-nutrient that blocks the absorption and digestion of carbohydrates. Complex oligosaccharide acarbose, retards the digestion of carbohydrates. It blocks the pancreatic action on amylase in the starch breakdown. Artificial inhibitors give rise to the effect like diarrhea, abdominal pain, and soft stools in colon. They discovered that the plant extract efficiently inhibit the alpha-amylase enzyme. The result suggest that the plant extract sufficiently blocks α-glucosidase and α-amylase enzymes in *in-vitro*. Therefore, possible antioxidant, anti-bacterial and anti-diabetic activities were exhibited in the crude leaves extract of *A. indica*; this accounts for its medicinal activities.

CONCLUSION: The current study reveals that the extract of *Azadirachta indica* consists of few medicinal and bio-active compounds. *Azadirachta indica* would be efficacious against drug-resistant microorganisms. It is one of the good sources of antioxidant compounds. These biologically active compounds are chemically assorted and structurally complex. Further research on secondary metabolites of plant extract are contemplated to advance new biologically active antimicrobial, antioxidant and anti-diabetic compounds. There is uprising engrossment in utilizing the plant extracts as therapeutic agents, so *A. indica* will serve as a major source for medicinal use in the pharmaceutical industry.

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CONFLICTS OF INTEREST: Nil

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