ISSN No:-2456-2165

Preliminary Studies and *In-vitro* Antioxidant Activity of Fruit-Seed Extracts of *Melia azedarach* Linn

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Abstract:- *Melia azedarach* is a medicinal plant utilized for the treatment of various disorders in Asian countries. The antioxidant potential of fruit-seeds from this plant was investigated using n-hexane, chloroform, methanol and aqueous extracts. Preliminary studies were performed on powder as well as on various extracts. An in vitro anti-oxidant activity was evaluated using DPPH, ABTS and TAC assays. All aforementioned extracts were rich in flavonoids and polyphenols. The aqueous extract (61.30 ± 3.55%) showed high DPPH scavenging activity followed by methanol (52.95% ± 0.53%), chloroform (52.61 ± 3.59%), and n-hexane extract (51.16 ± 1.70%). Similarly, all the extracts exhibited high ABTS scavenging activity. However, in TAC assay, chloroform extract (81.16 ± 1.09%) exhibited the highest inhibitory activity, followed by aqueous, methanol and n-hexane. Therefore, further isolation and characterization of the plant is required to explore the bioactive compounds for their anti-oxidant activity.

Keywords:- *Melia azedarach, physicochemical properties, phytochemical screening, anti-oxidant.*

I. INTRODUCTION

Oxidative stress is a predisposing factor that is caused by an imbalance equilibrium between production and accumulation of reactive oxygen species (ROS) in cells, resulting in the pathogenesis of acute as well as chronic disorders such as, atherosclerosis, aging, diabetes, immunosuppression and other neurodegenerative disorders (1). In living organisms, the ROS can be formed in various ways, including normal aerobic respiration and stimulated peroxisomes in polymorph nuclear leukocytes and macrophages (2). The role of free radicals and other ROS in disease pathology has been well documented in previous studies. The administration of natural antioxidants has been associated with the prevention of degenerative disorders such as neurological and cardiovascular disorders (3).The search for novel natural antioxidants of plant origin has ever since increased that might help prevent oxidative damage. Studies on medicinal plants have been indicated the presence of antioxidants such as, flavonoids, phenolics acids, proanthocyanidins, polyphenols and tannins which may contribute to prevention from various disorders (4). The ingestion of natural antioxidants with the multiple components source comprising of multiple components helps to counter the risk of oxidative stress-induced physiological malfunctions (5).

Melia azedarach belonging to *Meliaceae* family or more commonly known as Chinaberry tree has been used as folk remedies for the treatment of various diseases in India and Pakistan (6). Different parts of pants have been used in various ailments such ulcers, diabetes, leprosy, kidney stones and infectious diseases (7). The constituents recognized for their pharmacological activities are considered important for further investigation of the plant *M. azedarach* and various compositional patterns depending on the plant part, origin, extraction and analytical procedures have been reported (8). Alcoholic extracts of *Melia azedarach* leaves exhibit anti-oxidant activity in 2,2-diphenyl-2-picrylhydrazyl (DPHH) scavenging and FRAP assays (8-10). It exerts high scavenging activity due to the presence of the hydroxyl group in phenolic compounds. The ethyl acetate extracts also show positive effects in the metal-chelation assay (11). Hence, the current study was designed to evaluate the *in-vitro* antioxidant activity of different crude extracts of fruit-seed of *Melia azedarach* by using three *in-vitro* complementary assays: DPPH (1,1- diphenyl-2-picrylhydrazyl) free radical scavenging assay, ABTS (2,2`-azinobis-(3-ethylbensthiazoline-6-sulfonic acid) scavenging assay and molybdate assay.

II. MATERIAL AND METHODS

A. Plant collection

The fresh fruit-seeds were collected, separated, cleaned and air dried under shade for 2 weeks at room temperature. The plant material was then crushed into a fine powder and stored in an air-tight bag. It was then subjected to physicochemical and phytochemical screening.

B. Physicochemical studies/Proximate analysis

Proximate analysis of *Melia azedarach* fruit-seed powder was performed according to the method given in USP (2005), which is as follows:

a) Moisture content

Approximately 2 g powdered material was placed in a tarred china dish and put in an oven at 105°C. After 30 minutes, the china dish was taken out from the oven and placed in the desiccator for 10- to 15 minutes at room temperature. After cooling the contents, the weight of the china dish was measured. The weight of the content was recorded after every 30 minutes until the weight became constant. Then, the percentage of moisture content was calculated by using the following formula:

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Dry matter= weight of air-dried sample-weight of oven-dried sample
Moisture content (%) = (Dry matter/Weight of air-dried sample) x 100

b) Total ash content
2 g powdered material was taken in a tarred silica crucible. The material was then incinerated in a muffle furnace by gradually increasing the temperature to 625±25℃ until it became white presenting the absence of all carbon. Then cool the crucible in a desiccator at room temperature and measured the weight. Total ash contents were evaluated by using the following formula:
Total ash (%) = (weight of ash/weight of sample) x 100

c) Acid insoluble ash
Total ash which was previously obtained was boiled in 25ml of dilute HCL for about 5 minutes. The contents obtained were filtered through the ashless filter paper. The residue was washed with hot distilled aqueous. Then content was subsequently transferred into silica crucible, dried in an oven and incinerated in the muffle furnace at 625±25℃ until all carbon was removed. The crucible is allowed to cool in desiccator at the temperature and after that is weight is again measured. The acid-insoluble ash was evaluated by using following formula:
Acid insoluble ash (%) = (weight of ash/weight of the sample) x 100

d) Acid soluble ash
The percentage of acid soluble ash was assessed by calculating the difference of total ash and acid-insoluble ash.

e) Aqueous-insoluble ash
Total ash was boiled for 5 minutes in 25ml of distilled aqueous. It was then filtered through ashless filter paper. Residues were washed with hot distilled aqueous and contents are transferred into the silica crucible. The crucible was then into the muffle furnace at 625±25℃ until all carbon was removed. After that, the crucible was taken out and allowed to cool in the desiccator. Aqueous-insoluble ash was determined by the following equation:
Aqueous insoluble ash (%) = (weight of ash/weight of sample) x 100

f) Sulphated ash
2 g of powdered material was taken into tarred silica crucible. The contents were moistened with concentrated sulphuric acid and ignited gently until white fumes were stopped evolving. The procedure was repeated twice and then crucible placed in muffle furnace at 625 ± 25 ℃ until the content became free of carbon. The percentage of sulphated ash was evaluated by using the following formula:
Sulphated ash (%) = (weight of sulphated ash/weight of the sample) x 100

g) Aqueous-soluble extractives
5 g powdered material was macerated in 100ml of double strength chloroform aqueous (99 ml aqueous + 1 ml chloroform) for 24 hours with continuous stirring. The sample was then filtered via filter paper and the filtrate (25 ml) was dried in an oven at 105℃. The weight was again measured after drying and percentage of aqueous-soluble extractive was determined by using the following formula:
Alcohol soluble extractives (%) = (weight of dried filtrate/weight of air-dried sample) x 100

h) Alcohol soluble extractives
5 g powdered material was macerated in 100 ml of ethyl alcohol for about 24 hours with continuous stirring. The sample was filtered and the filtrate (25 ml) was dried in an oven at 105 C. After drying weight of the content was again measured and the percentage of alcohol soluble extractive was calculated by using the following formula:
Alcohol soluble extractives (%) = (weight of dried filtrate/weight of air-dried sample) x 100

C. Extraction procedure
A 50g of powdered material was taken and contents were separated sequentially using Soxhlet apparatus. All the extracts were dried with the help of rotary evaporator, keeping the temperature less than the boiling point of the respective solvent. All extracts were collected in previously weighed, cleaned and labeled storage vials, and allowed to dry in the oven at 40℃ until the solvent disappeared.

a) Percentage yield
The percentage yield of each extracts was calculated by reference to the initial weight of the fruit-seed powder.

D. ATR spectroscopy of crude powder
Fruit-seed powder was subjected to ATR analysis. The material was pressed directly on to the diamond crystals having high refractive indices FTIR spectra was obtained in range 4000- 400cm^{-1}

E. Estimation of polyphenols
Total polyphenol contents of fruit-seed of Melia azedarach were evaluated according to protocol prescribed by Singleton and Slinkard(12). A methanolic solution of Gallic acid (mg/ml) of different concentrations was used as a standard to plot standard calibration curve. Accordingly, 200ul of standard/test solution was taken into falcon tubes. A 200ul of Folin-Ciocalteu reagent was added in the respective falcon tubes and mixed the solution homogenously. After five minutes, 1ml of 7.5% of sodium carbonate was added into the falcon tubes and the final volume was made up to 3ml with methanol. Blank was treated like test solution/standard with the exception of standard or test solution which is replaced by methanol. The reaction mixture was incubated at room temperature for about 2 hours and absorbance was then measured at 760nm. Standard curve of gallic acid was plotted and polyphenol content was determined from the calibration curve of the

ISSN No:-2456-2165

standard. The phenolic contents were expressed as mg/g of gallic acid equivalent by the linear regression equation.

F. Estimation of total flavonoids

Pavun *et al* (2018) method was used for the determination of the total flavonoids with little modification (13). A methanolic solution of quercetin (mg/ml) of different concentration was used as a standard to plot the standard calibration curve. A reaction mixture contains 200ul of standard/test solution, 100ul 10% aluminium nitrite and 100ul of 1M potassium acetate and final volume was made up to 5ml with distilled aqueous. Falcon tubes were then incubated for 30 minutes at room temperature and absorbance was measured at 415nm. The standard curve of quercetin was plotted and flavonoid content was determined from the calibration curve of standard.

G. Anti-oxidant activity

a) DPPH (1,1- diphenyl-2-picrylhydrazyl) free radical scavenging assay

The DPPH free radical scavenging activity was evaluated spectro metrically by *Kumar et al*. method with little modification(14). Accordingly, 1ml of the extract was mixed with 1ml of 0.004% (w/v) methanolic solution of DPPH was added in the test tubes and volume was made upto 3 ml with methanol. It was then incubated for 30 minutes in a dark place at room temperature. The absorbance was measured at 517nm using a UV-Visible single beam spectrophotometer. Ascorbic acid was used as standard. The control was treated like a test solution with the except test solution/standard which was replaced by the solvent used.The DPPH free radical scavenging assay was then determined by using following formula and expressed as percentage (%) scavenging of DPPH radical,

$$\text{Percentage inhibition}(\%) = \frac{(\text{Absorbance of control} - \text{Absorbance of sample}) \times 100}{\text{Absorbance of control}}$$

b) ABTS Assay

The ABTS assay was estimated by *Ohikhena et al*. method with little modification(15). ABTS oxidizes into radical cations when reacted with potassium persulphate. Accordingly, 7 mM ABTS solution and 2.45mM potassium persulphate solution was mixed in equal amount and allowed it to stand for 18 hours at room temperature in the dark place before use. The resultant solution was then diluted with methanol to achieve an absorbance of 0.700±0.020 at 734nm at 30 ℃. Then, 1ml of plant extracts of different concentrations was mixed with 1ml of the resultant solution and incubated for 7 minutes in a dark place and then absorbance of the reaction mixture was measured at 734nm. Ascorbic acid was used as standard and treated as test solutions. Control was prepared by replacing the standard/extracts with the solvent used and treated like test solution. The assay was then calculated by using the following formula;

$$\text{Percentage inhibition}(\%) = \frac{(\text{Absorbance of control} - \text{Absorbance of sample}) \times 100}{\text{Absorbance of control}}$$

c) Total antioxidant capacity (TAC) assay

Molybdate assay was evaluated according to the method of *Ohikhena et al*. with little modification (15). Accordingly, 300ul extract of different concentrations was mixed with 3 ml of reagent (0.6 M sulphuric acid, 4mM ammonium molybdate and 28mM sodium phosphate) in falcon tubes. It was then covered with aluminium foil and place in a aqueous bath at 95℃ for 90 minutes. The solution was allowed to cool at room temperature and absorbance was then measured at 765 nm. A mixture contains distilled aqueous instead of standard/test solution served as control. Ascorbic acid was used as a standard. The percentage inhibition was then calculated by using the following formula;

$$\text{Percentage inhibition}(\%) = \frac{(\text{Absorbance of sample} - \text{Absorbance of control}) \times 100}{\text{Absorbance of sample}}$$

H. Statistical analysis

All the tests were performed in triplicates and the means were calculated. All the values were expressed as means ± standard deviation (SD).

III. RESULTS AND DISCUSSION

A. Physicochemical properties

Physicochemical evaluation of the crude drugs is an important parameter to identify the improper handling of drugs or adulteration (16). The results of physicochemical parameters of *Melia azedarach*fruit-seed were shown in Table 1.

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Physicochemical Properties	Content (% w/w)
Moisture content	6.5 ± 0.14
Total Ash	5.0 ± 0.12
Acid-soluble ash	1.5 ± 0.05
Acid-insoluble ash	3.5 ± 0.31
Aqueous-insoluble ash	4.2 ± 0.06
Sulphated ash	3.5 ± 0.38
Aqueous-soluble extractives	5.5 ± 0.09
Alcohol-soluble extractives	6.5 ± 0.04

Table 1: Physicochemical properties of fruit-seed of *Melia azedarach*

Moisture content determination is an important factor to assess the stability of the crude drug as well as powders. Crude drugs with higher moisture content lead to chemical degradation and microbial growth (17). Moisture content should be minimal so that material remains stable for a longer period of time. The general requirement for crude drug is that moisture content should be less than 14% w/w. Total moisture content is found to be 6.5% which indicates that it is within the specified range. Ash values and soluble extractive values were determined to assess the quality and purity of the fruit-seed powder of *Melia Azedarach*. Ash value is evaluated to identify any foreign matter like oxalate, silicate, carbonate etc. According to British Pharmacopeia (BP), the total value of ash should not be greater than 20%. Our study reported that the total ash of selected plant material was under specified range. Extractive values are calculated to identify adulterated or exhausted drugs (16). High alcohol and aqueous-soluble values suggested the presence of polar substances like tannins, phenols, glycosides etc.

B. Percentage Yield

Sequential extraction of *Melia azedarach* fruit seed powder was done by five different solvents (least polar to most polar) which included: n-hexane, petroleum ether, chloroform, methanol and water.The percentage yield of plant extracts using different solvents shows that water extract has maximum percentage yield as compared to other solvent extracts as shown in figure 1.

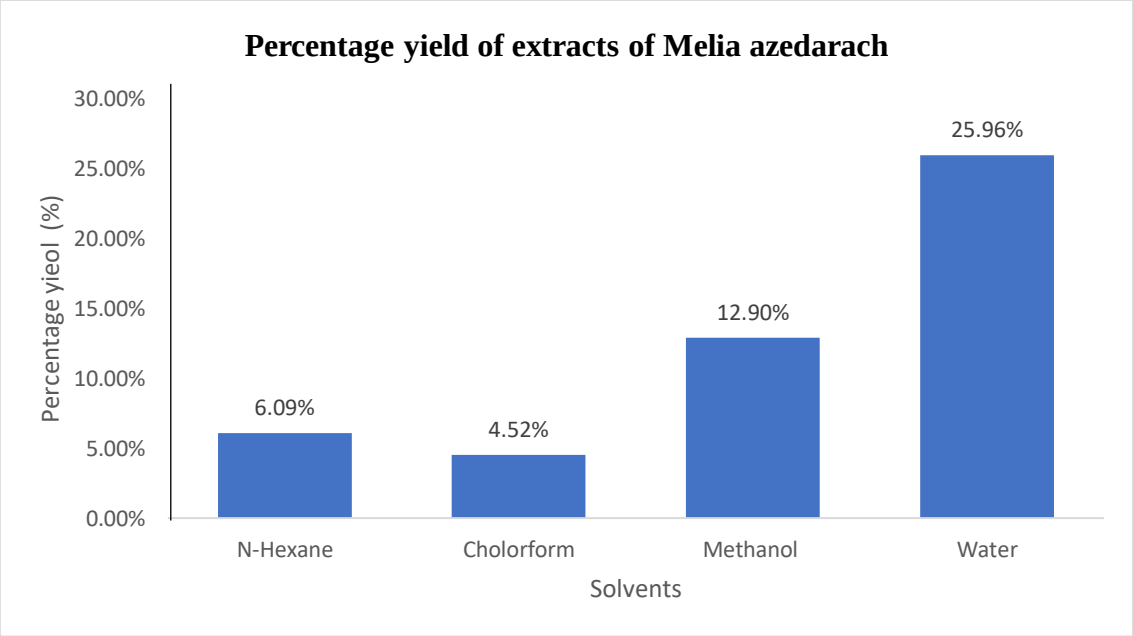

Fig. 1: Percentage yield of different extracts of Melia azedarach fruit seed powder

C. Estimation of polyphenols and flavonoids

The analysis of total polyphenols and total flavonoids was expressed in table 1. The results revealed that the fruit-seed part of *Melia azedarach* contains the maximum quantity of polyphenols in chloroform, followed by n-hexane, aqueous and methanol respectively. The chloroform extract showed the highest quantity of flavonoids while methanolic extract showed the minimum quantity of the flavonoids.

Secondary metabolites play an important part in fighting against pests and various diseases such as cancer, cardiovascular, autoimmune and infectious diseases (18). A variety of secondary metabolites was found in plants that exhibit pharmacological and therapeutic activities. Polyphenols and flavonoids are two important components of secondary metabolites that showed antioxidant as well as anti-inflammatory effects (19). These components are also involved in the prevention of various acute and chronic disorders such as arthritis, cancer and cardiovascular diseases (19).

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Extracts	Total Polyphenols (mg/g)	Total Flavonoids (mg/g)
n-Hexane	28.23±0.46	95.33±0.51
Chloroform	77.65±0.53	74.71±0.67
Methanol	10.31±0.33	22.13±0.38
Aqueous	42.06±0.69	23.45±0.76

Table 1: Estimation of total polyphenols and flavonoids.

D. Antioxidant activity

It is well-known that free radicals cause the auto-oxidation of unsaturated lipids in food(20). On other hand, antioxidants cause the breakdown ofthe free-radical chain of oxidation and donate hydrogen ions, resulting in a stable compound, which does not further involve in the oxidation of lipids and fatty acids(21). The anti-oxidant activity of various plant extracts at various concentration of 10ug/ml to 500ug/ml was estimated by three *in-vitro* methods i.e. DPPH assay, ABTS assay, and TAC assay.

a) DPPH assay

DPPH assay is the most common approach used for the determination of the antioxidant potential in a compound, a plant extract, or other biological sources. DPPH is stable nitrogen-centered free radical that scavenged effectively in the presence of an anti-oxidant agent and strong absorbance was showed at 517nm(22). The scavenging activity of various extracts (aqueous, methanol, chloroform and n-hexane) of *Melia azedarach* were determined by comparing percentage inhibition of DPPH radicals caused by the presence of anti-oxidant molecules in extracts with standard that is ascorbic acid. The absorbance was decreased with increase in concentration, resulting in the increase in percentage inhibition of DPPH radicals. The aqueous extract exhibited the highest inhibitory activity (61.30±3.55) on DPPH radical, followed by chloroform extract (52.61±3.59%), methanol extract (52.95±0.53%) and n-hexane extract (51.16±1.70%). At 500ug/ml, all extracts have above 50% inhibitory activity. Based on IC50, the order of scavenging activity of the various plant extracts was; Chloroform>N-hexane>Methanol>Aqueous (Figure 1).

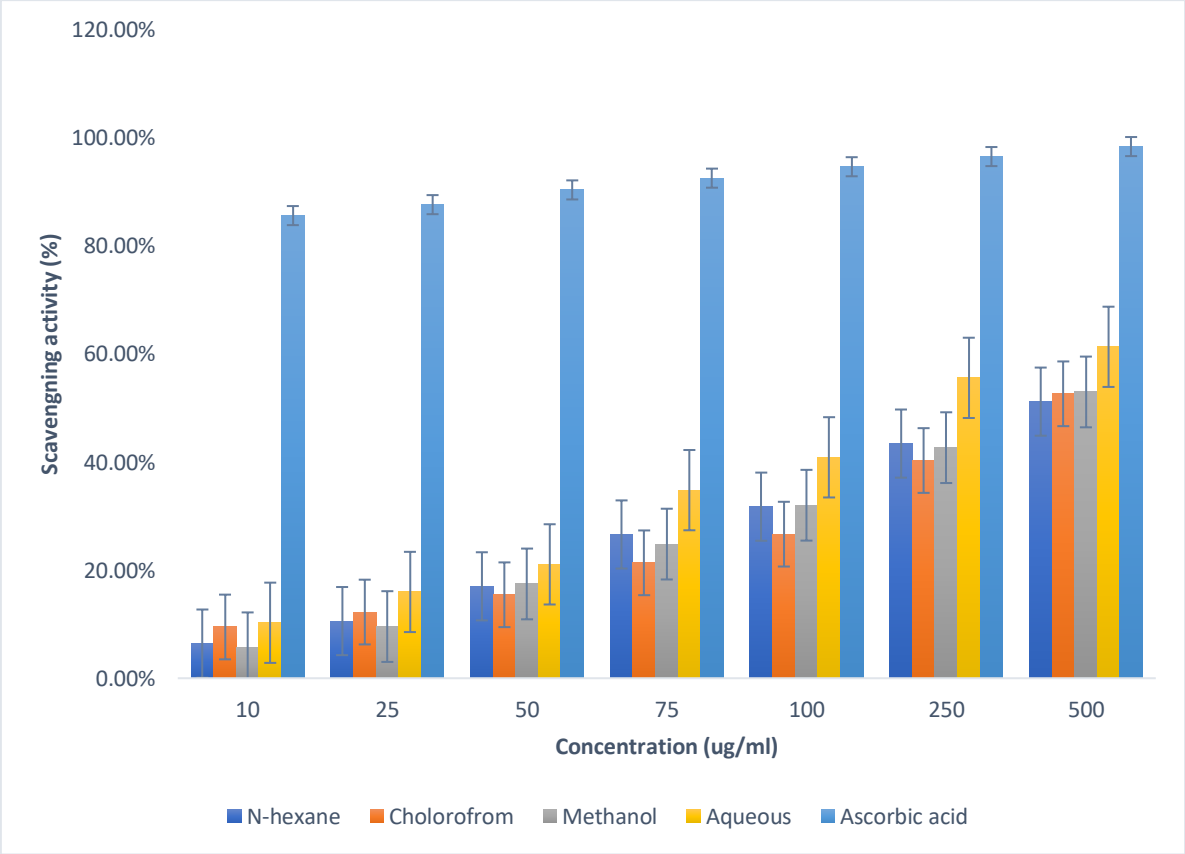

Fig. 1: DPPH scavenging activity of different extracts of Melia azedarach

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b) ABTS assay

ABTS assay is also one of the most common approaches used for the evaluation of antioxidant potential in the extracts, lead compounds and other biological sources. 2,2`-azinobis-(3-ethylbensthiazoline-6-sulfonic acid) (ABTS) releases free radical cation that react with every lipophilic as well as hydrophilic substance(23).The results were summarized in table 4.2.All plant extracts exhibited good ABTS radical scavenging activity. The absorbance of reaction mixtures was decreased with an increase in concentration. At concentration of 500ug/ml, aqueous extract (71.06±3.82) exhibited highest ABTS radical activity, followed by chloroform (69.60±1.99), methanol (61.06±2.63) and n-hexane (50.88±2.18). All extracts showed inhibitory activity on ABTS over 50%. Based on IC_{50}, the order of scavenging activity of the various extracts and standard was; ascorbic acid>aqueous>chloroform (Figure 2).

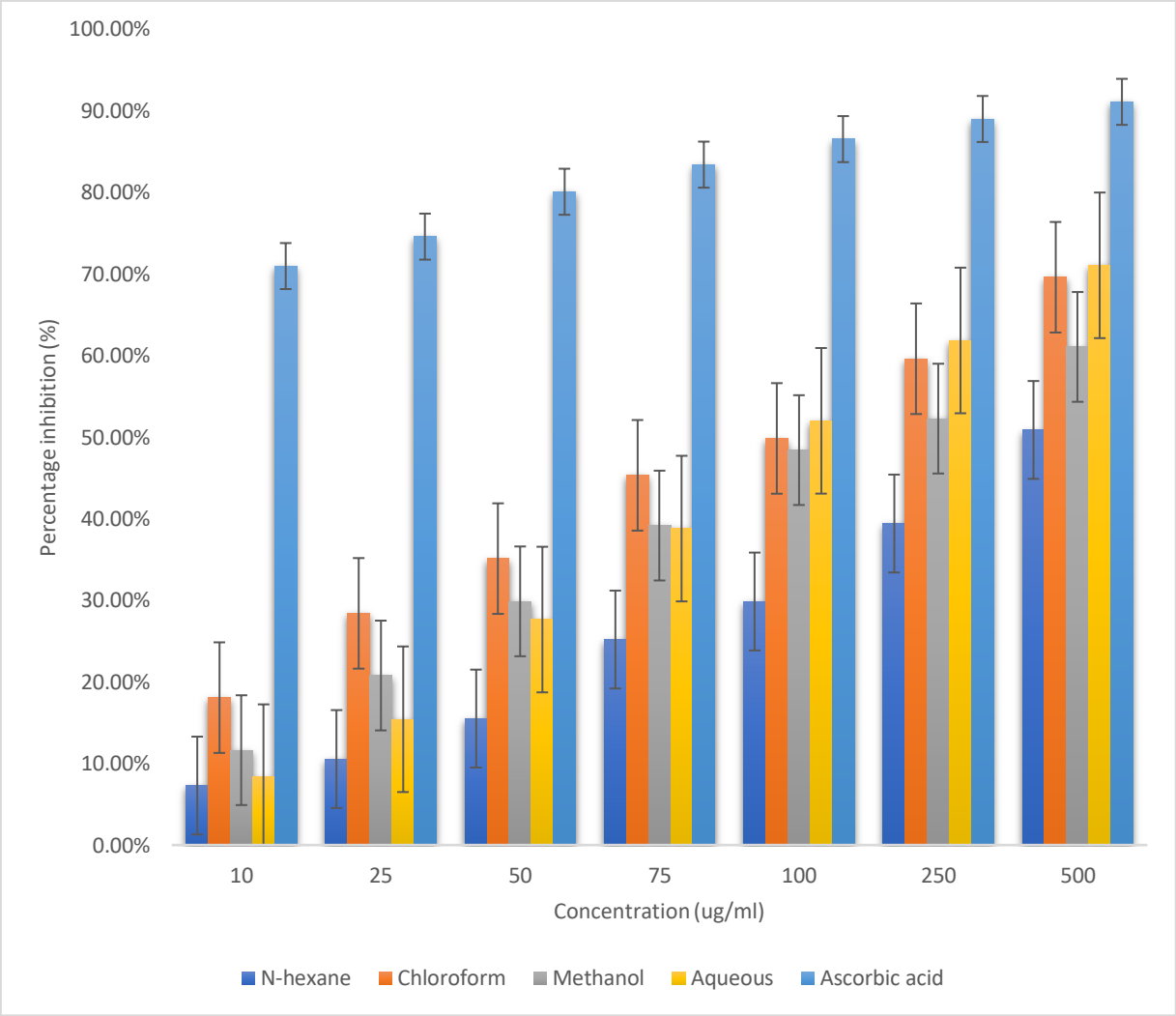

Fig. 2: ABTS scavenging activity of different extracts of Melia azedarach

The total antioxidant capacity (TAC) of *Melia azedarach* extract of various concentrations was determined by phosphomolybdenum assay (Figure 3). The absorbance of reaction mixtures was increased with the increase in concentration, resulting an increase in antioxidant capacity. The chloroform extract showed the highest inhibition ranged from 10.96±2.37 at 10ug/ml to 81.16±1.09 at 500ug/ml; aqueous extract ranged from 28.01±1.93% at 10ug/ml to 72.18±3.37% at 500ug/ml; methanolic extract had a TAC from 22.91±1.11% at 10ug/ml to 65.01±3.65% at 500ug/ml; n-hexane extract was from 6.44±1.18% at 10ug/ml to 59.03±2.77% at 500ug/ml. The IC50 value of various plant extracts was in order; n-hexane>methanol>aqueous>chloroform (Table 4.4).

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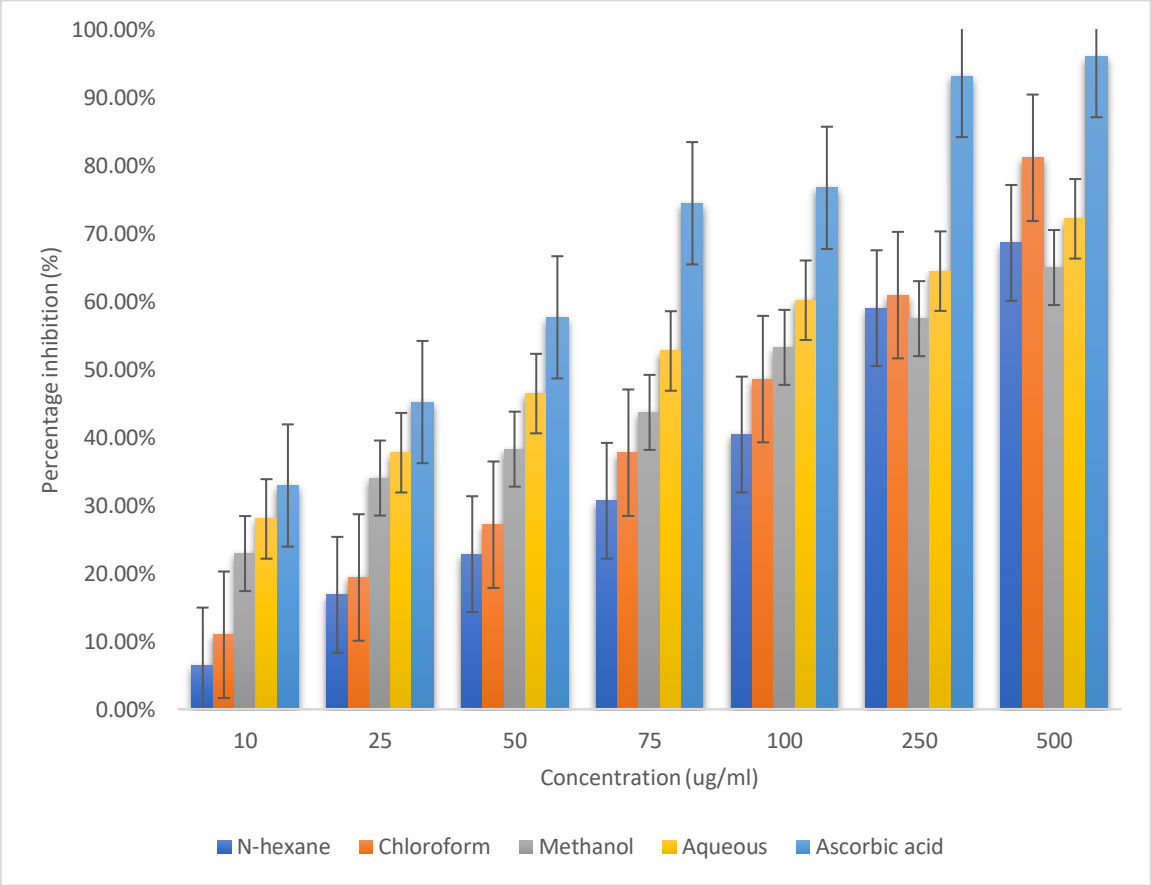

Fig. 3: Total antioxidant capacity assay of different extracts of Melia azedarach

Types of extracts	DPPH		ABTS		TAC	
	IC_{50} (ug/ml)	R^2	IC_{50} (ug/ml)	R^2	IC_{50} (ug/ml)	R^2
N-hexane	417.81	0.8075	445.98	0.8450	272.58	0.8326
Chloroform	426.90	0.9303	214.61	0.7684	214.40	0.8668
Methanol	406.69	0.8323	292.47	0.6833	216.02	0.7193
Aqueous	304.94	0.7705	237.81	0.7282	121.06	0.6849

IC_{50}: Concentration (ug/ml) required to inhibit 50% or more scavenging activity, R^2=Co-efficient of determination, DPPH= 1,1-diphenyl-2-picrylhydrazyl, ABTS= 2,2`-azinobis-(3-ethylbensthiazoline-6-sulfonic acid), TAC= Total antioxidant activity.

Table 2: IC_{50} values of the different extracts of *Melia azedarach*.

IV. CONCLUSION

The current study supports the idea that the fruit-seed of *Melia azedarach* is a rich source of natural anti-oxidants. The aqueous extract exhibited the highest inhibitory activity in two *in-vitro* assay i.e. DPPH and ABTS assay while in TAC assay, chloroform exhibited the highest inhibitory activity. Therefore, further isolation and characterization of the plant is required to explore the bioactive compounds for their anti-oxidant activity.

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