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IN-VITRO EVALUATION OF ANTIOXIDANT ACTIVITIES OF *OLDENLANDIA CORYMBOSA* L. (SYN. *HEDYOTIS CORYMBOSA*) FRACTIONATED EXTRACTS IN VARIOUS SOLVENTS

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Keywords:

Herbal Medicine, Antioxidants, Free Radical, Soxhlet Extraction, Fractionation, Reducing Power

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ABSTRACT: Antioxidants are the substance having the potential to quench free radicals and significantly delay or inhibit oxidation of the substrate, thus protect biological systems against potentially harmful effects of free radicals; in low concentrations. The present study was aimed to evaluate the *in-vitro* antioxidant activity of *Oldenlandia corymbosa* L., a locally available medicinal plant of Assam, used as vegetable in diet and traditional medicine for liver disease and jaundice. The dried powdered plant material was extracted with 80% ethanol by Soxhlet extraction and was used for the fractionation in different solvents (methanol, petroleum ether, and ethyl acetate). The fractionated extracts were subjected to the analysis of *in-vitro* antioxidant activity with DPPH (2,2, Diphenyl-2-picryl hydrazyl), hydroxyl radical, nitric oxide, reducing power, and phosphomolybdenum assays for total antioxidant capacity. Ascorbic acid was used as standard at concentrations (20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml) dissolved in ethanol. Samples were prepared in the same manner. Results from *in-vitro* experiments revealed the significantly ($p<0.05$) high antioxidant activity in hydro-ethanolic extract than methanol, petroleum ether, and ethyl acetate extracts when compared with a standard antioxidant, ascorbic acid. *O. corymbosa* contains some important groups of phytochemicals having exogenous antioxidant properties. Our findings provide scientific support for ethnomedicinal uses of *O. corymbosa* to cure jaundice and liver-related ailments and indicate a promising antioxidant potential of this plant for the development of herbal therapy against various oxidative stress-related diseases.

INTRODUCTION: Free radicals play an important role in the life and death of cells. If the endogenous antioxidants fail to overcome the production of the reactive metabolites, then exogenous antioxidants would be necessary to balance redox status.

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Dietary sources, including plants, herbs, spices, vitamins, and herbal extracts, play an important role in coping up with these problems [1].

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) play an important role in many biological processes and are involved in host defense, overproduction of these species such as hydroxyl radical, hydrogen peroxide, superoxide anions, and nitric oxide contribute to the immuno-pathology of a wide variety of conditions including inflammatory diseases, cancer, atherosclerosis, diabetes mellitus, hypertension, AIDS, and aging [2, 3].

Oxidative stress constitutes a disturbance caused by an imbalance between the generation of free radicals and antioxidant system, which causes damage to biomolecules. Thus, in turn, may lead to the occurrence of many chronic degenerative diseases. Therefore, it is very important to know the functioning of those endogenous (and exogenous) antioxidants systems to prevent such diseases. Such systems are intrinsic in cells at intracellular and extracellular levels and act together with the dietary exogenous antioxidants [4]. Antioxidants based drugs for the treatment of various pathological diseases have gained attraction in clinical as well as research areas.

Phytochemicals such as flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins, isocatechins, epicatechin, *etc*. are being used in clinical and preclinical trials and exhibit a great importance in research area because of useful medical applications of plant-derived drugs [5, 6]. *O. corymbosa* is an annual, terrestrial, dichotomous, slender ascending herb commonly known as parpata, pitpapdo, wild pepper, and diamond flower is a common wild, non-tuberous flowering species found in many parts of India including north-east India and other tropical regions of the world. It is found widely in the Brahmaputra valley region of the northeastern part of India. It is a plant that belongs to the *Rubiaceae* family and popularly known as bon jaluk or sarpajibha in Assamese.

Traditionally, the plant leaves and stems are generally used as leafy vegetables in the diet. Daily use of its juice prevents stomach problems arising due to gastric. It acts as a blood purifier and helps in normal blood circulation process and effective medicine for jaundice and liver diseases. It can kill intestinal worms (anti-helminthic). Juice from this plant is used in gall bladder disease and gonorrhea [7, 8]. It is also known to act against tumors of the digestive tract lymphosarcoma and carcinoma of the liver and larynx. It is also active against appendicitis, hepatitis, pneumonia, cholecystesis, urinary infection, cellulitis, and snakebite. Chinese folk medicine describes the plant to treat skin sores, ulcers, sore throat, bronchitis, gynecologic infections, and pelvic inflammatory [9, 10, 11, 12]. Previous studies reported *O. corymbosa* methanolic extract is to be anti-hepatotoxic [13], antioxidant potential [14, 15, 16], hepatoprotective [17], antimicrobial [18] and strong analgesic effect [19] in its leaf, stem and root parts, but the antioxidant property in different fractionated solvent extracts in-vitro has not yet been reported.

Thus, the present work is focused on the antioxidant potential of fractions of the main crude hydro-ethanolic extract of *O. corymbosa* in three different solvents *in-vitro*.

FIG. 1: *OLDENLANDIA CORYMBOSA* L. (SYN. *HEDYOTIS CORYMBOSA*)

MATERIALS AND METHODS: The experiments were performed in the year 2017-19 at Department of Biotechnology, Gauhati University, Assam.

Plant Material: The whole plant parts of *Oldenlandia corymbosa* for the current study was collected from the roadside and field area of Gauhati University campus and roadside area of Mangaldai, Darrang, Assam during the month of November-December and authenticated by the Department of Botany, Gauhati University (Specimen Accession No. 18672, Ref. No:- *Herb*./GUBH/2019/132).

Extraction Procedure:

Preparation of Sample: The aerial parts and roots of *O. corymbosa* were washed thoroughly with tap water separately and then rinsed with distilled water. Leaves, stems, and roots of the plants were dried at room temperature separately without direct exposure to sunlight. When sufficiently dry, they were grounded to powder. The powder then extracted with the following method.

Soxhlet Extraction: For Soxhlet extraction, 50 gm of powdered sample was taken in 300 ml of the 80% ethanol in a Soxhlet apparatus at 60 °C until

the color of the solvent becomes colorless. The organic layers were filtered in Whatman filter paper no. 1, and the solvent was evaporated under a vacuum rotary evaporator to obtain a gummy crude extract. The concentrated ethanolic extract was then preserved in a beaker and covered with aluminum foil and stored in a refrigerator at 4 °C for further experiments.

Fractionation of Crude Hydro-ethanolic Extract for *in-vitro* Antioxidant Activity Analysis: Hydro-ethanolic crude extract obtained from Soxhlet extraction was used for fractionation. 2 gm crude extracts were dissolved in 20 ml of petroleum ether and stirred in magnetic stirrer for 1 h. The supernatant was collected by decantation, and the solvent was evaporated under a vacuum rotary evaporator to obtain a gummy crude extract. The remaining precipitate was dissolved in 20 ml ethyl acetate and stirred in magnetic stirrer for 1 h. The whole process was repeated by changing the solvent to methanol. All three extracts (petroleum ether, ethyl acetate, and methanol) were collected and stored in the refrigerator at 4 °C for *in-vitro* antioxidant analysis.

Chemicals: 0.1mM 2,2-diphenyl-1pikryl-hydrazyl ($C_{18}H_{12}N_5O_6$) [DPPH], ethanol, methanol, ascorbic acid, 0.2M Phosphate Buffer pH=6.6, 50mM Phosphate Buffer pH=7.4, Phosphate Buffer Saline, 1% potassium ferricyanide, 10% Trichloroacetic Acid (TCA), 10 mM Ferric Chloride ($FeCl_3$), 1% $FeCl_3$ Solution, 1mM Ethylenediaminetetraacetic acid (EDTA), 10mM Hydrogen Peroxide (H_2O_2), 10mM Deoxyribose, 0.5% Thiobarbituric acid (TBA), 10mM Sodium Nitroprusside, 0.6M sulphuric acid, 28mM Sodium phosphate, 4mM Ammonium Molybdate, 1% sulphanilic acid ($C_6H_7NO_3S$), 3% Phosphoric Acid, 0.1% NEDD (N-1-Naphthylethylenediamine dihydrochloride, $C_{12}H_{14}N_2$), 0.1N HCl, 0.1N NaOH, 5% Sodium Nitrite ($NaNO_2$), 10% Aluminium Chloride, Quercetin, 1M Sodium Hydroxide, Folin Ciocalteu Reagent (FCR), 20% Sodium Carbonate, Gallic Acid Monohydrate, Hydrochloric Acid.

Determination of Total Flavonoids by Aluminium Chloride Colorimetric Method: The total content of flavonoid was determined by the method of Chang *et al*. [20] The reaction mixture consists of 1ml of extract and 4 ml of distilled water was taken in a 10 ml of the test tube. 0.3ml of 5% Sodium Nitrite ($NaNO_2$) was treated, and after 5 minutes, 0.3 ml of 10% Aluminium chloride was mixed. After 5 min, 2ml of 1M sodium hydroxide was treated and distilled to 10ml with distilled water. A set of reference standard solution of Quercetin (20, 40, 60, 80, and 100 µg/ml) was prepared in the same manner. The absorbance for test and standard solution were determined against the reagent blank at 510 nm with a UV-Visible double beam spectrophotometer (Shimadzu UV1800). All the tests were performed in triplicates. Flavonoid contents were determined from the standard curve and were expressed as Quercetin equivalent (mg/g of the extracted compound) by the following formula-

$$\text{Total Flavonoid content} = C \times V / M$$

Where, C = Concentration of the extract from the calibration curve in mg/ml, V = Volume of the extract in ml, M = the weight of the extract in gm.

Determination of Total Phenolics by Folin–Ciocalteu method: Total phenolics content was determined by the method of Kaur *et al*. [21] Briefly, 200 µL of crude extract (1mg/mL) were made up to 3 mL with distilled water, mixed thoroughly with 0.5 mL of Folin–Ciocalteu reagent for 3 min, followed by the addition of 2 mL of 20% (w/v) sodium carbonate. A set of reference standard solutions of Gallic acid (20, 40, 60, 80, and 100µg/ml) were prepared in the same manner. The mixture was allowed to stand for a further 60 min in the dark, and absorbance was measured at 650 nm. The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of Gallic acid equivalent per g dry weight by the following formula-

$$\text{Total Phenolic content} = C \times V / M$$

Where, C = Concentration of the extract from the calibration curve in mg/ml, V = Volume of the extract in ml, M = the weight of the extract in gm.

***In-vitro* Antioxidant Assay:**

DPPH Test (Free Radical Scavenging Property): Free Radical Scavenging Property was determined by the preparation of a stock solution of DPPH (0.1mM) in ethanol (39.4 mg in 1 liter). 5 ml of DPPH solution was added to 1 ml of different

extract solution of different concentrations (10-100 µg/ml) and incubated for 30 min. Absorbance was measured at 517 nm against reagent blank and compared with the standard of the same concentration. The activities of the samples are measured in terms of percent inhibition (IC_{50}) and calculated by the following formula:-

Percent (%) inhibition = A - B × 100 / A …… (a)

Where, A = Optical density of the blank, B = Optical density of the sample

Antioxidant activity is expressed as IC_{50}. IC_{50} is the concentration in µg/ml of extract that inhibits the formation of DPPH radical by 50%. The IC_{50} value was calculated by plotting a graph of standard ascorbic acid with percent inhibition on the y-axis and concentration on the x-axis.

Reducing Power Assay: Reducing power property was determined by preparing various concentrations of *O. corymbosa* different solvents to extract in ethanol (10-100µg/ml) and mixed with 2.5 ml of 0.2 M phosphate buffer pH 6.6 and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C in a hot water bath for 20 min. 2.5 ml of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 10 min. 2.5 ml of the upper layer was taken and mixed with 2.5 ml of distilled water, and then 0.5 ml of freshly prepared ferric chloride solution was added. Absorbance was measured at 700 nm and compared with the standard of the same concentrations. Reducing the ability of the standard ascorbic acid and samples were determined by plotting a graph with absorbance in y-axis and concentration in x-axis.

Hydroxyl Radical Scavenging Property: Hydroxyl radical scavenging property was determined by preparation of a reaction mixture by adding 0.1 ml of 1mM EDTA, 0.01 ml of 10mM $FeCl_3$, 0.1 ml of 10 mM H2O2, 0.36 ml of 10 mM Deoxyribose, 1 ml of extract solution of different concentration (10-100µg/ml) of different solvent extracts, which were mixed in ethanol, 0.33 ml of 50mM Phosphate buffer pH 7.4. The reaction was then incubated at 37 °C for 1 h. 1 ml of 10% TCA, and 1 ml of 0.5% TBA was added. The reaction mixture was then heated at 95 °C for 15 min and then cooled. Absorbance was taken at 532 nm. The hydroxyl radical scavenging activity of the extract was reported as % inhibition deoxyribose degradation. Percentage inhibition was calculated by the formula (a) as described in the DPPH test. The IC50 value was calculated by plotting a graph of standard ascorbic acid with percent inhibition on the y-axis and concentration on the x-axis.

Nitric Oxide Scavenging Property: The scavenging property of nitric oxide was determined by preparing 10mM Sodium nitroprusside in phosphate buffer saline. The sodium nitroprusside solution was mixed with different concentrations of different solvent extracts solutions (10-100µg/ml) in ethanol and incubated at 25 °C for 1hr and 30 min. 1.5 ml of incubated solution was taken, and it 1.5 ml of Griess reagent was added. Absorbance was then taken at 546 nm. Percentage scavenging property was calculated by the formula (a) as described in the DPPH test. The IC50 value was calculated by plotting a graph of standard ascorbic acid with percent inhibition on the y-axis and concentration on the x-axis.

Phosphomolybdate Assay for Total Antioxidant Capacity: To determine total antioxidant capacity (TAC) of *O. corymbosa* extracts as per phospho-molybdate assay by Prieto *et al*., [22] and the procedure described by Jan *et al*., 23 was used with slight modification. For sample preparation, a stock solution of 1mg plant extract was dissolved in 1 mL methanol and sonicated for 5 min to get a homogeneous mixture. Ascorbic acid was used as a standard. A stock solution of ascorbic acid (1mg/ml) was prepared in distilled water, from which dilutions were made ranging from 200 µg/ml to 1000 µg/ml. In a test tube, 300 µL plant extract was mixed with 3 mL phosphomolybdate reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The test tube was covered with aluminum foil and incubated at 95 °C for 90 min. The mixture was then allowed to reach room temperature when its absorbance was recorded at 765 nm. Blank was run using the same procedure but containing an equal volume of methanol in place of the plant sample. The antioxidant capacity was reported as µg of ascorbic acid equivalents (AAE) per mL by the following formula-

TAC = C × V / M…….. (b)

Where, TAC = Total Antioxidant Capacity, C = Concentration of the extract from the calibration curve in mg/ml, V = Volume of the extract in ml, M = the weight of the extract in gm.

Statistical Analysis: All the results of antioxidant assay were done in triplicates and were expressed as mean ± S.D. The significant differences between different solvent extracts were determined by one-way analysis of variance (ANOVA) with a significance level at 0.05. Two-tailed unpaired Student's t-test was used to test the significance of differences between the results obtained for the extract and standard. A probability value of less than 0.05 was considered significant. The analyses were done by using Microsoft office excel software, Windows-7 Ultimate.

RESULTS:

Total Flavonoid Content of *Oldenlandia corymbosa*: Total flavonoid content of the Hydro-ethanolic extract and fractions in various solvents was determined in terms of µg of Quercetin Equivalent (QE) per gm of the extract, and the results are shown in **Fig. 2**. The total flavonoid content decreases in the following order: hydro-ethanol > methanol > petroleum ether > ethyl acetate.

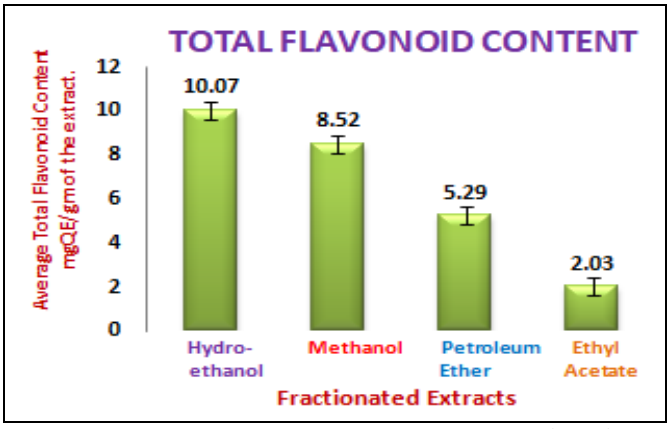

FIG. 2: TOTAL FLAVONOID CONTENT (TFC) OF FOUR DIFFERENT SOLVENT EXTRACTS OF *O. CORYMBOSA*

Total Phenolic Content of *Oldenlandia corymbosa*: The hydro-ethanolic extract and fractions in various solvents of *O. corymbosa* were subjected to evaluation of total phenolic content, and the results are shown in **Fig. 3**. In this study, the hydro-ethanolic extract showed the highest phenolic content (7.01mg of Gallic Acid Equivalent/GAE per gm of the extract), while the ethyl acetate fraction had the lowest value (1.42 mg of GAE per gm of the extract). The phenolic content in various solvents decreases in the order: hydro-ethanol > methanol > petroleum ether > ethyl acetate.

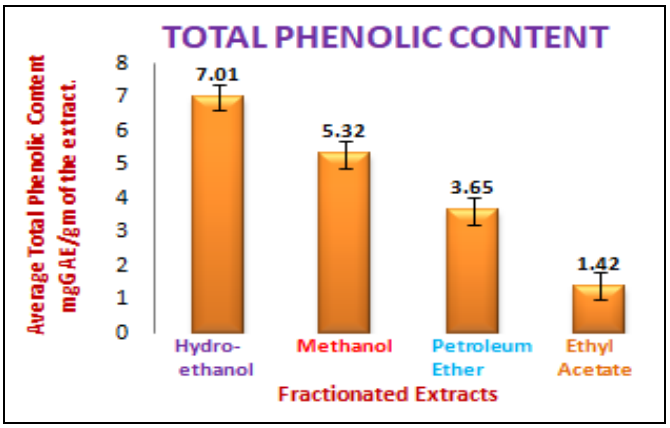

FIG. 3: TOTAL PHENOLIC CONTENT (TPC) OF FOUR DIFFERENT SOLVENT EXTRACTS OF *O. CORYMBOSA*

***In-vitro* Antioxidant Assay:**

DPPH Test (Free Radical Scavenging Property): The hydro-ethanolic extract showed a significant ($p<0.05$) increase in free radical scavenging activity than methanol, ethyl acetate, and petroleum ether extracts. The IC_{50} value of hydro-ethanol, methanol, ethyl acetate, and petroleum ether extracts were found as 58.26 µg/ml, 59.89 µg/ml, 78.96 µg/ml and 108.06 µg/ml respectively as compared to ascorbic acid standard IC_{50} of 41.11 µg/ml **Fig. 5**. The hydro-ethanolic extract showed a significant increase in free radical scavenging activity as compared to methanol, petroleum ether, and ethyl acetate extract **Fig. 4**.

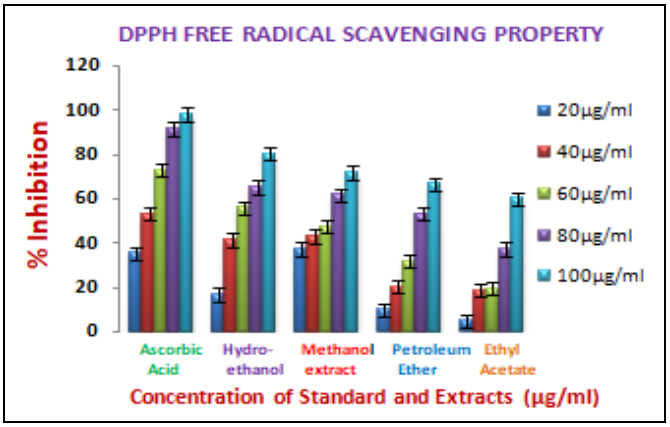

FIG. 4: % INHIBITION OF ASCORBIC ACID AND FOUR FRACTIONATED EXTRACT OF *O. CORYMBOSA* IN DIFFERENT CONCENTRATIONS (µg/ml) SHOWING SIGNIFICANT ($p<0.05$) INCREASE IN % INHIBITION OF DPPH RADICAL SCAVENGING PROPERTY IN HYDRO-ETHANOLIC EXTRACT THAN METHANOL, PETROLEUM ETHER, AND ETHYL ACETATE FRACTIONS

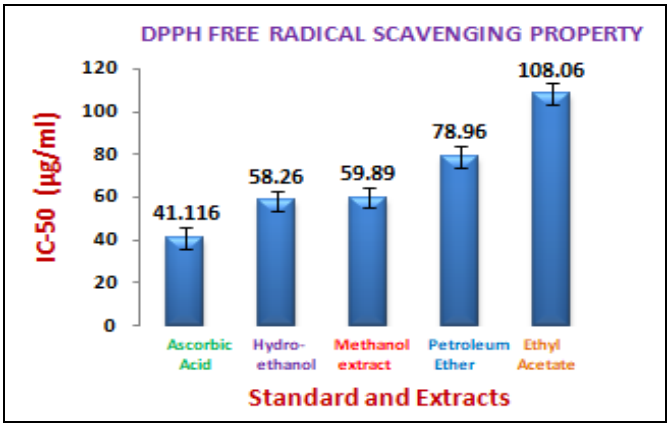

FIG. 5: 50% INHIBITION CONCENTRATION (IC_{50} IN µg/ml) OF FOUR EXTRACTS OF *O. CORYMBOSA* AND ASCORBIC ACID FOR DPPH FREE RADICAL SCAVENGING PROPERTY

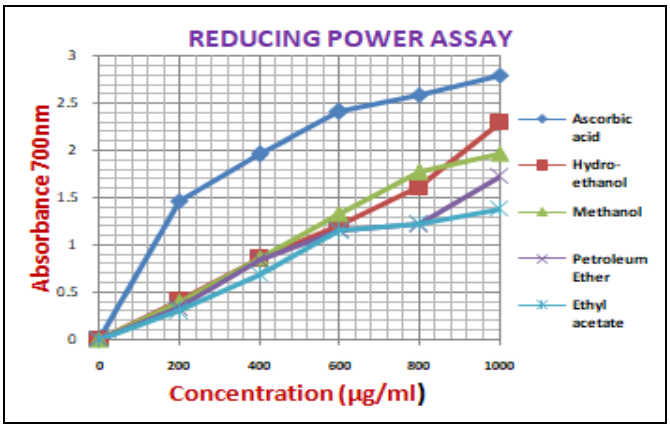

FIG. 6: ABSORBANCE OF FOUR EXTRACTS OF *O. CORYMBOSA* AND ASCORBIC ACID AGAINST DIFFERENT CONCENTRATIONS OF THE STANDARD AND EXTRACT FOR REDUCING POWER ASSAY

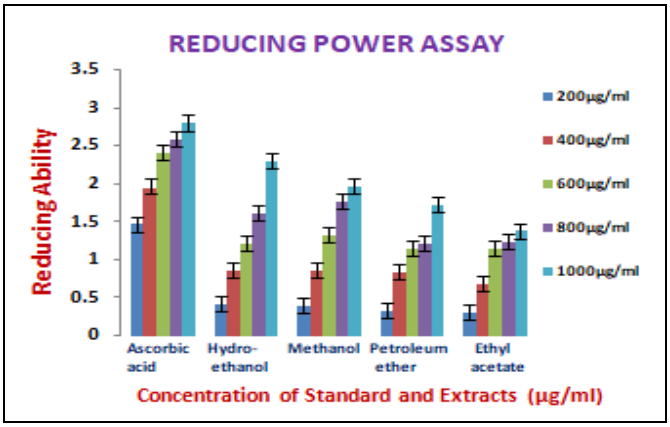

FIG. 7: REDUCING ABILITY OF FOUR EXTRACTS OF *O. CORYMBOSA* AND ASCORBIC ACID IN DIFFERENT CONCENTRATIONS SHOWING HIGHEST REDUCING ABILITY IN HYDRO-ETHANOLIC EXTRACT AS COMPARED TO STANDARD ASCORBIC ACID THAN METHANOL, PETROLEUM ETHER AND ETHYL ACETATE EXTRACTS.

Reducing Power Assay of *O. corymbosa*: The reductive property was measured by observing the ability of the antioxidant to transform potassium ferricyanide to potassium ferrocyanide, which then reacts with ferric chloride to form a ferric ferrous complex that has an absorption maximum at 700 nm. The absorbance values of four extracts were plotted in the Y-axis, and different concentrations of the extracts (200, 400, 600, 800, 1000 µg/ml) plotted in the X-axis **Fig. 6**. From the graph, reducing the ability of extracts was compared with ascorbic acid standard. The hydro-ethanolic extract showed a significant ($p<0.05$) increase in reducing capability than methanol, ethyl acetate, and petroleum ether extracts **Fig. 7**.

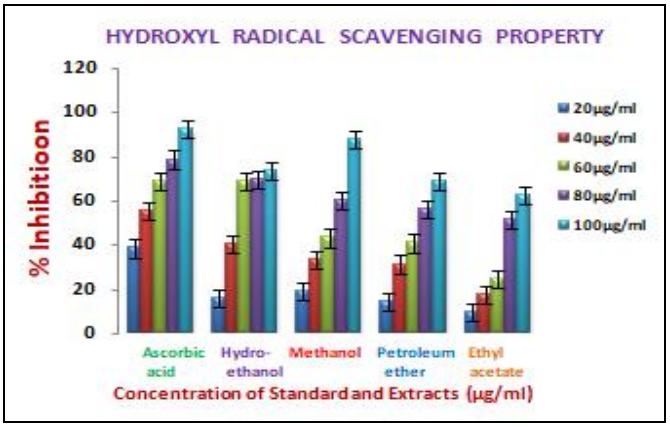

FIG. 8: % INHIBITION OF ASCORBIC ACID AND FOUR FRACTIONATED EXTRACT OF *O. CORYMBOSA* IN DIFFERENT CONCENTRATIONS (µg/ml) SHOWING HIGHEST % INHIBITION IN HYDRO-ETHANOLIC EXTRACT AS COMPARED TO ASCORBIC ACID STANDARD THAN METHANOL, PETROLEUM ETHER AND ETHYL ACETATE FRACTIONS

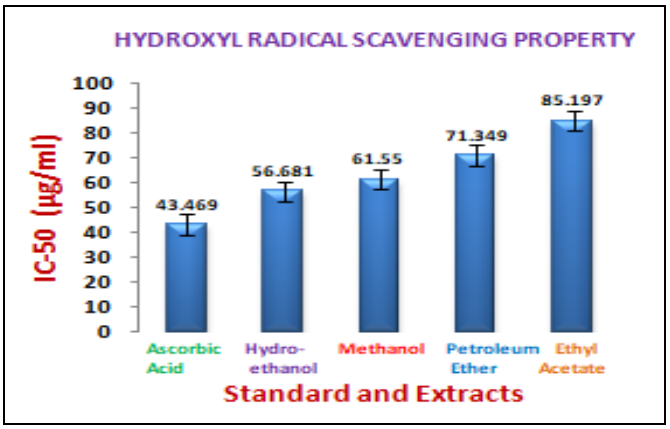

FIG. 9: 50% INHIBITION CONCENTRATION OF FOUR EXTRACTS OF *O. CORYMBOSA* AND ASCORBIC ACID FOR HYDROXYL RADICAL SCAVENGING PROPERTY

Hydroxyl Radical Scavenging Property of *O. corymbosa*: The reaction generates hydroxyl radicals which degrade deoxyribose using Fe^{2+} salts

as an important catalytic component. The hydro-ethanolic extract showed a significant ($p<0.05$) increase in hydroxyl radical scavenging activity than methanol, ethyl acetate, and petroleum ether extracts. The IC_{50} value of hydro-ethanol, methanol, ethyl acetate, and petroleum ether extracts were found to be as 56.6 µg/ml, 61.5 µg/ml, 71.3 µg/ml and 85.1 µg/ml respectively as compared to ascorbic acid standard IC_{50} of 43.4 µg/ml **Fig. 9**. The hydro-ethanolic extract showed a significant increase in hydroxyl radical scavenging activity, as compared to methanol, petroleum ether and ethyl acetate extracts **Fig. 8**.

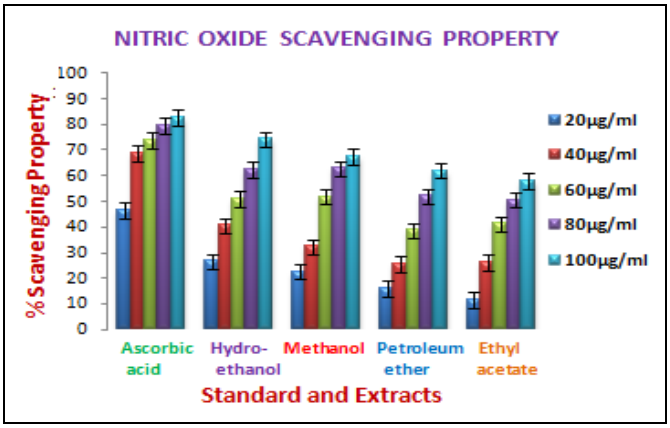

FIG. 10: % SCAVENGING PROPERTY OF ASCORBIC ACID AND FOUR FRACTIONATED EXTRACT OF *O. CORYMBOSA* IN DIFFERENT CONCENTRATIONS (µg/ml) SHOWING SIGNIFICANT (P<0.05) INCREASE % SCAVENGING ACTIVITY IN HYDRO-ETHANOLIC EXTRACT THAN METHANOL, PETROLEUM ETHER AND ETHYL ACETATE FRACTIONS

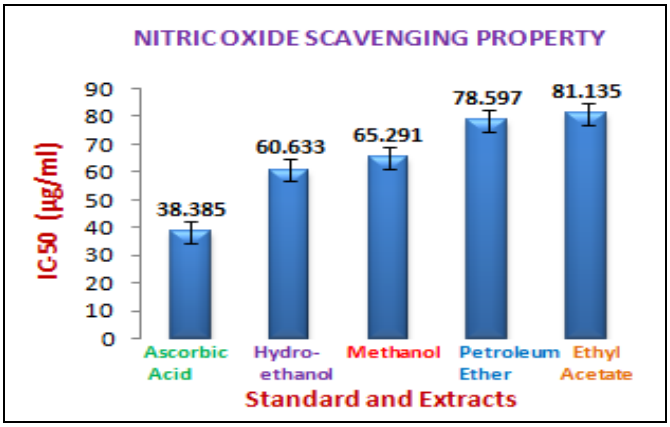

FIG. 11: 50% INHIBITION CONCENTRATION OF FOUR EXTRACTS OF *O. CORYMBOSA* AND ASCORBIC ACID FOR NITRIC OXIDE SCAVENGING PROPERTY

Nitric Oxide Scavenging Property of *O. corymbosa*: Sodium nitroprusside at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. The IC_{50} value of hydro-ethanol, methanol, ethyl acetate, and petroleum ether extracts were found to be as 60.6 µg/ml, 65.2 µg/ml, 78.5 µg/ml and 81.1 µg/ml respectively as compared to ascorbic acid standard IC_{50} of 38.3 µg/ml **Fig. 11**. The hydro-ethanolic extract showed significant increase in nitric oxide scavenging property as compared to methanol, petroleum ether and ethyl acetate extracts **Fig. 10**.

Phosphomolybdenum Assay for Total Antioxidant Capacity of *O. corymbosa*: This assay is based on the reduction of molybdenum (VI) to molybdenum (V) which takes place in the presence of a reducing agent (antioxidant). The product is a green phosphomolybdate (V) complex whose formation is monitored with a spectrophotometer. The assay is often used to estimate the total antioxidant activity of a sample, and the results are expressed in terms of ascorbic acid equivalents (AAE). The Phosphomolybdenum assay for the total antioxidant capacity of *O. corymbosa* was studied in four different solvent extracts *i.e.*, hydro-ethanol, methanol, ethyl acetate, and petroleum ether.

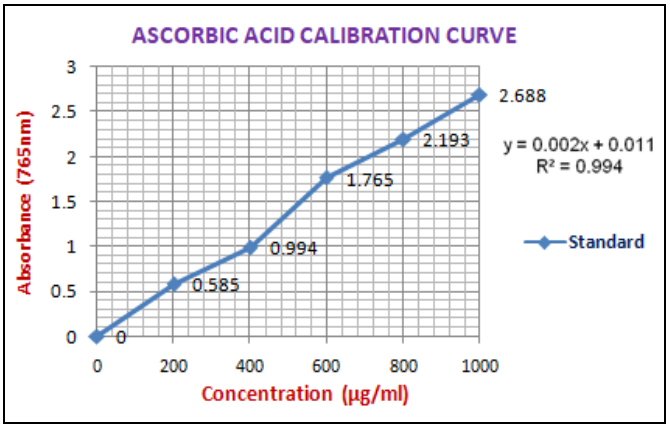

FIG. 12: STANDARD CURVE OF ASCORBIC ACID OF DIFFERENT CONCENTRATIONS FOR PHOSPHO-MOLYBDENUM ASSAY

The concentrations of the four different extracts (µg/ml) were calculated from the ascorbic acid standard calibration curve **Fig. 12** and converted into mg/ml, which was put into the formula (b) for total antioxidant capacity determination. The total antioxidant activity of *O. corymbosa* was found to be significantly high in hydro-ethanolic extract (107.33 mg/gm AAE) as compared to methanol

(67.55 mg/gm AAE), petroleum ether (62.51 mg/gm AAE) and ethyl acetate (59.92 mg/gm AAE) extracts (**Fig. 13** and **Fig. 14**).

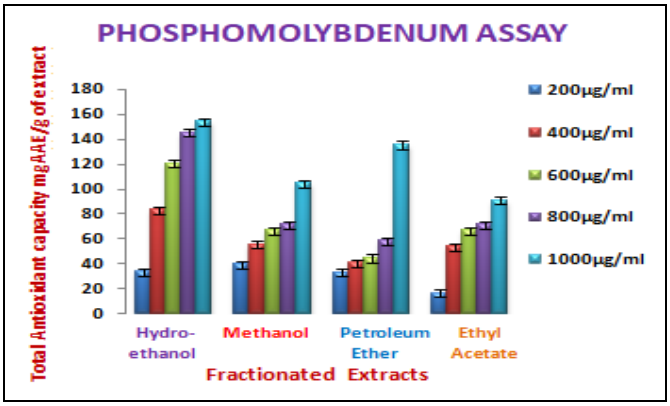

FIG. 13: TOTAL ANTIOXIDANT CAPACITY OF FOUR FRACTIONATED EXTRACTS OF *O. CORYMBOSA* SHOWING HIGHEST ANTIOXIDANT CAPACITY IN HYDRO-ETHANOLIC EXTRACT THAN METHANOL, PETROLEUM AND ETHYL ACETATE EXTRACTS

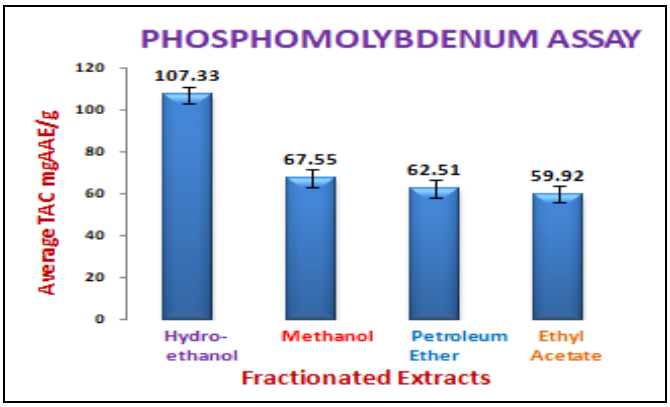

FIG. 14: AVERAGE TOTAL ANTIOXIDANT CAPACITY OF FOUR FRACTIONATED EXTRACTS OF *O. CORYMBOSA*

DISCUSSION: Antioxidants are chemical compounds with monohydroxy/polyhydroxy phenols; which works to slow down the lipid peroxidation. These compounds have low activation energy to donate hydrogen atom and, therefore, cannot initiate the second free radicals [24]. Any defect in the proper removal of ROS/RNS can cause serious damage to the body and, if not repaired over a period of time, can cause serious tissue injury, ultimately inducing tumor formation. Oxidation is one of the most important routes for producing free radicals in food, drugs, and even in living systems. The imbalance between oxidants and antioxidants is one of the main reasons for many chronic diseases [25].

Total Flavonoid Content of *O. corymbosa*: Almost every group of flavonoids has the capacity to act as a powerful antioxidant that can protect the human body from free radicals and reactive oxygen species [26]. Flavonoids have been shown to have a wide range of biological and pharmacological activities such as anti-inflammatory activity, enzyme inhibition, antimicrobial activity, oestrogenic activity, anti-allergic activity, antioxidant activity, vascular activity and cytotoxic antitumor activity [27]. The total flavonoid content of the methanol extract of *O. Corymbosa* was found to be 8.52 mgQE/g of extract. From the previous study, the total flavonoid content of methanolic extract of *Oldenlandia corymbosa* was reported to be as 4.4mg/gm by [28]. This difference of flavonoid content may be attributed to the diverse geographical location and due to the soil quality. *O. Corymbosa* contains a significantly high amount of flavonoids in hydro-ethanolic extract (10.07 mg of QE), which indicate the presence of important antioxidant compounds because flavonoid constitutes a major portion of the antioxidants.

Total Phenolic Content of *O. corymbosa*: Phenolic compounds are responsible for their chemopreventive properties (*e.g.*, antioxidant, anticarcinogenic, or antimutagenic and anti-inflammatory effects) and also contribute by inducing apoptosis by arresting cell cycle, regulating carcinogen metabolism and ontogenesis expression, inhibiting DNA binding and cell adhesion, migration, proliferation or differentiation, and blocking signaling pathways [28]. The total phenolic content of methanol extract of *O. Corymbosa* was found to be 5.32 mgGAE/g of extract. From the previous study total phenolic content of methanol extract of *Oldenlandia corymbosa* was reported to be 11.6 mg/gm by Yadav *et al*. [29] The hydro-ethanolic extract showed significant ($p<0.05$) high amount of TPC (7.01 mgGAE), which is high enough to act as an antioxidant *in-vitro*.

***In-vitro* Antioxidant Assay:**

DPPH test (Free Radical Scavenging Property): Radical scavenging activities are very important to prevent the deleterious role of free radicals in oxidative stress diseases. The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples, including plant extracts [30]. The DPPH is a stable free radical (DPPH·) that accepts

an electron or hydrogen radical to form diamagnetic molecule [31, 32]. During the process, the purple color of the radical turns pales yellow. The DPPH radical shows maximum absorbance at 517 nm. The % inhibition of DPPH radical scavenging property in the hydro-ethanolic extract is significantly higher than petroleum ether and ethyl acetate extracts, but methanol extract showed almost same % inhibition since hydro-ethanol, and methanol extract has no significant ($p>0.05$) difference in DPPH radical scavenging property, which may be due to same range of polarity index of the solvents (water-10.2 + ethanol-5.1 and methanol-5.2). The ability of the samples to scavenge DPPH free radical decreases in the order of hydro-ethanol > methanol > petroleum ether > ethyl acetate. Thus, as the polarity index of the solvent decreases, the scavenging properties of the extracts are also decreased.

The % inhibition of hydro-ethanolic extract shows a strong correlation ($R = 0.997$ and $R = 0.984$) with total flavonoids content and total phenolic content. The IC_{50} is the concentration of the extract required to give 50% inhibition of the free radical activity of DPPH. The IC_{50} of hydro-ethanol (58.26 µg/ml) and methanol (59.89µg/ml) extracts were almost same, but the petroleum ether (78.96 µg/ml) and ethyl acetate extracts (108.06 µg/ml) showed high IC_{50} value, *i.e.,* low antioxidant property.

Reducing Power Assay of *O. corymbosa*: Depending on the reducing power of the testing compound, the yellow color of the Fe(III) changes to Fe(II), Perl's Prussian blue which is a measure of the ability of the antioxidant (or a reducing agent) to transform potassium ferricyanide to potassium ferrocyanide, which then reacts with ferric chloride to form a ferric-ferrous complex that is monitored spectrophotometrically (λ_{max} 700 nm). As the concentration of the extract increases, the transformation of potassium ferricyanide to ferric-ferrous complex increases. Thus absorption value also increases, which indicates the increase in antioxidant compounds in increasing concentration of the extracts. Higher the absorbance value, higher the reducing ability. The hydro-ethanolic extract shows significantly ($p<0.05$) high reducing ability than three other extracts when compared with standard ascorbic acid. The ability of the samples to reduce Fe(III) to Fe(II) decreases in the order of Hydro-ethanol > Methanol > Petroleum ether > Ethyl acetate.

Hydroxyl Radical Scavenging Property of *O. corymbosa*: Hydroxyl radicals (.OH) are the major active oxygen species causing oxidation of polyunsaturated fatty acid in food and enormous cellular and tissue damage [33]. The effect of the different solvent extracts of *O. corymbosa* on hydroxyl radicals generated by Fe^{3+} ions was measured by determining the degree of deoxyribose degradation, an indicator of Thiobarbituric acid–malonaldehyde (TBA-MDA) adduct formation. As shown in **Fig. 7**, the hydro-ethanolic extract inhibited significantly ($p>0.05$) hydroxyl radical-induced deoxyribose degradation in a concentration-dependent manner. The value of 50% inhibition concentration (IC_{50}) of the hydro-ethanolic extract is almost near (56.68 µg/ml) to the standard ascorbic acid (43.46 µg/ml). The antioxidant components in the plant extracts competed with deoxyribose against the -OH radical generated from the Fe^{3+} dependent system and prevented the reaction. The antioxidant(s) in the extract could be acting as chelators of the Fe^{3+} ions in the system, thereby preventing them from complexing with the deoxyribose, or simply donating hydrogen atoms and accelerating the conversion of H_2O_2 to H_2O [34]. The observed ability of the extracts to scavenge or inhibit -OH radical indicates that the extracts could significantly inhibit lipid peroxidation since -OH radicals are highly implicated in peroxidation.

Nitric Oxide Scavenging Property of *O. corymbosa*: Nitric oxide (NO^-) released from Sodium Nitroprusside (SNP) has a strong NO^+ character, which can alter the structure and function of many cellular components. NO scavenging activity is leading to the reduction of the nitrite concentration in the assay medium. The NO scavenging capacity was concentration-dependent with 100 µg/ml scavenging most efficiently. The Hydro-ethanolic extract in SNP solution significantly inhibited ($p<0.05$) the accumulation of nitrite, a stable oxidation product of NO^+ liberated from SNP in the reaction medium with time compared to the standard Ascorbic acid. The toxicity of NO^+ increases when it reacts with superoxide to form the peroxynitrite anion

(ONOO), which is a potential strong oxidant that can decompose to produce OH^- and NO_2 [35].

The present study shows that a hydro-ethanolic extract of *O. corymbosa* has a potent nitric oxide scavenging activity than methanol, petroleum ether, and ethyl acetate extracts.

Statistical Analysis: There is a correlation between % Inhibition and total flavonoid, total phenolic content of all the extracts. From the correlation analysis, it was found that there is a highly strong correlation between % Inhibition of DPPH (R=0.99 and 0.98), NO (R=0.98 and 0.99), OH (R=0.98 and 0.94) radical activity of hydro-ethanolic extract with total flavonoid content and total phenolic content, which indicates that, a hydro-ethanolic extract of *O. corymbosa* contains some important polyphenolic compounds (in high amount) having potent exogenous antioxidant property.

From the t-Test of IC_{50} of all the extracts for DPPH, NO and OH radical activity revealed that there is no significant ($p<0.05$) difference between them in terms of % inhibition. Thus, the rate of increasing antioxidant activity from ethyl acetate extract to hydro-ethanolic extract was almost the same and concentration dependant.

CONCLUSION: *Oldenlandia corymbosa* is an important medicinal plant of India, has potent antioxidant properties. It has a significant amount of flavonoid and phenolic (polyphenolic) compounds in the hydro-ethanolic extract. The methanolic extract was also found to be most active. These differences in antioxidant activities are due to the efficiency of the extraction of phytochemicals by solvents with respect to different polarity index and other chemical properties. The high antioxidant activity in the hydro-ethanolic extract was may be due to the presence and effect of a particular bioactive compound or a group of phytochemicals, but further study is required to identify and isolate principal bioactive compounds present in *O. corymbosa* that needs more elaborate and extensive study.

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