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PHYTOCHEMICAL SCREENING AND *IN-VITRO* ANTIOXIDANT ACTIVITY OF *KNEMA ATTENUATA* (HOOK. F. & THOMSON) WARB STEM BARK EXTRACT

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Keywords:

Knema attenuata, Folk medicine, Phytochemical screening, Antioxidant, DPPH

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ABSTRACT: The plant *Knema attenuata* or *Myristica attenuata* (Myristicaceae) is an endemic tree species found in the Western Ghats. Traditionally, the stem bark of *K. attenuata* is used in folk medicine without any scientific information available for the same. Phytochemical screening was carried out for ethanolic stem bark extract of *K. attenuata* to investigate the antioxidant potential of the extract. The antioxidant activity of the extract was estimated employing *in-vitro* models: DPPH radical scavenging assay, total antioxidant activity, nitric oxide scavenging assay and reducing power activity. The extract was found to be rich in phenolics, alkaloids, and flavonoids. Quantitative estimation of extract demonstrated higher levels of phenolics and flavonoids. The extract exhibited significant radical scavenging activity and a total antioxidant capacity compared with that of ascorbic acid (Standard) which confirms the use of the species in folk medicine.

INTRODUCTION: Free radicals are reactive species with an unpaired electron that are produced during cell metabolism as a result of the endogenous enzymatic or non-enzymatic reactions [1, 2]. They behave as oxidants or reductants either by donating an electron to or accepting one from other molecules. Free radicals being highly reactive, target biologically relevant macromolecules like lipids, proteins, DNA, *etc*. leading to cell damage and homeostatic imbalance [1, 3]. Antioxidants are compounds those control free radical generation or their access to important biological sites either by neutralizing them or by scavenging them. Sometimes an excessive generation of free radicals may occur in stressful conditions like trauma, infections, toxins, *etc*., due to the increased synthesis of radical generating enzymes [2].

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This disturbs the free radical-antioxidant balance and exerts oxidative stress leading to various diseases like cancer, hypertension, hepatotoxicity *etc*. [3, 4] This oxidative stress has to be resolved through external supply of substances which possess antioxidant properties. This is possible to an extent by synthetic antioxidant drugs. Since these synthetic drugs can cause adverse side effects; a better option will be to focus on the screening of antioxidants from natural sources [5]. Phyto-constituents present in various plant parts like phenolics; flavonoids *etc*. are reported to possess high antioxidant property which plays an important role in the adsorption or neutralization of free radicals [6].

The plant *Knema attenuata* or *Myristica attenuata* (Myristicaceae), commonly known as wild nutmeg is an endemic tree species found in Western Ghats [7]. The bark of this tree has been used in folk medicines as an ingredient of 'Ashwagandhadi nei' (medicated ghee), used for the treatment of spleen disorders, breathing disorders and impaired taste sensation [8]. A lignan "attenuol" has been isolated from the stem bark of *Knema attenuata*; closely

related to lignans isolated from plants of Myristicaceae family[9]. Lignans possess antioxidant, liver protection, anti-inflammatory and anti-cancer properties [10]. However, there are no reports on phytochemical studies or pharmacological screening in the stem bark of *Knema attenuata* till date. Therefore, the present study was undertaken to screen the bark extract phytochemically and to estimate the quantitative antioxidant potential of ethanolic stem bark extract of *K. attenuata*.

MATERIALS AND METHODS:

Plant Material: The plant species for the proposed study *Knema attenuata*, was collected from Kerala Forest Research Institute, Peechi, Thrissur in January 2015. The taxonomic authentication of species was carried out by Dr. V. B. Sreekumar, Scientist (Botany Department), Kerala Forest Research Institute, Peechi, Thrissur. The fresh stem barks were collected from the plant and spread in trays and air dried for three weeks. The dried stem bark was powdered and sieved (sieve no: 44) for extraction [11].

Preparation of the Extract: 50 g of stem bark powder was packed in a thimble and loaded in a Soxhlet apparatus. It was subjected to continuous extraction with 300 ml 95% ethanol. After completing extraction of the bark powder, 80% of the solvent was recovered by using a rotary evaporator (at 40 °C) and remaining solvent was removed by heating on a water bath. The dried extract was stored in a desiccator (to keep it free from moisture) over activated silica gel until needed [12].

Qualitative Phytochemical Screening: Ethanolic stem bark extract of *K. attenuata* was subjected to preliminary phytochemical screening using standard procedures to estimate the presence of phytoconstituents like carbohydrates, glycosides, alkaloids, flavonoids, steroids, *et*c. [13]

Quantitative Phytochemical Analysis:

Determination of Total Phenolic Content: Total phenolic content in the extract was determined by the Folin-Ciocalteu method using gallic acid as standard [14]. To 1 ml of 1 mg ml^{-1} extract solution, 0.25 ml of Folin- Ciocalteu reagent and 1.25 ml of 20% sodium carbonate solution were added and kept for 40 min at room temperature. After the reaction period, the contents were mixed and measured the blue color at 765 nm using Jasco 530 UV-Visible spectrophotometer. A calibration curve of standard gallic acid was prepared and using that the total phenolic content present in the extract was determined as µg gallic acid equivalent.

Determination of Total Flavonoid Content: Total flavonoid content in the extract was determined by the aluminium chloride method using quercetin as reference compound [15]. A stock solution of 1 mg/ml extract was prepared, from which 1 ml was pipetted out. To this 4 ml, water was added followed by 0.3 ml of 5% sodium nitrite. After 5 min, 0.3 ml of 10% aluminum chloride solution was added, and at the 6^{th} min, 2 ml of 1M sodium hydroxide was added. The total volume was made up to 10 ml with distilled water. A blank was prepared without the addition of aluminum chloride solution. The solutions were mixed well, and the absorbance was measured against the blank at 510 nm using UV-Visible spectrophotometer. A calibration curve of standard quercetin was prepared and using that total flavonoid content present in the extract was estimated.

***In-vitro* Antioxidant Studies:**

DPPH Radical Scavenging Activity: The assay was based on the decoloration of purple DPPH radical by the extract, indicating its antioxidant property [16]. 3 ml of DPPH solution was added to 1 ml various concentration (100, 250 & 500 µg ml^{-1}) of extracts or standard solution of ascorbic acid (100, 250 & 500 µg ml^{-1}). The mixture was shaken and incubated in darkness at room temperature for 30 min, and the absorbance was measured at 517 nm by using a spectrophotometer. Ascorbic acid was used as a standard. 3 ml DPPH reagent and 1 ml methanol was used in control. Ethanol was used as a blank. All experiments were done in triplicate and meant values were recorded. Scavenging activity of DPPH free radical in percentage was calculated using the following formula;

Percentage inhibition = [(OD of control-OD of test) / OD of control] × 100

Where OD is the optical density.

Total Antioxidant Activity: Determination of Total anti-oxidant activity was based on phospho-molybdenum method [17]. 0.3 ml of ethanolic extract of 100 µg ml^{-1} concentration was combined with 3

ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes containing reaction mixture were incubated at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the solutions was measured at 695 nm against a blank. A typical blank solution contained 3 ml of reagent solution and 0.3 ml of the same solvent used for the sample, and it was incubated under the same conditions as that of the sample. Ascorbic acid was used as a standard, and a calibration curve was obtained using various concentrations of ascorbic acid. The experiment was conducted in triplicates and value was expressed as equivalents of ascorbic acid.

Nitric Oxide Free Radical Scavenging Activity: Evaluation of $NO^{\bullet}$ scavenging activity was based on the method described by Griess in 1879.1810 mM of sodium nitroprusside was prepared freshly and sodium nitroprusside in phosphate buffer saline (2.5 ml) was mixed with 0.5 ml of different concentrations (50, 100, 500 µg ml^{-1}) of extract in ethanol and incubated at 25 °C for 3 h. After incubation, 0.5 ml is pippeted out and mixed with 2 ml of Griess reagent (or 1 ml of sulphanilic acid and after 5 min 1 ml of naphthyl ethylenediamine dihydrochloride) and incubated for 30 min.

The absorbance of the pink color developed during diazotization of nitrite with sulphanilamide and its subsequent coupling with naphthyl ethylenediamine dihydrochloride was observed at 550 nm on a spectrophotometer. Different concentrations of ascorbic acid (50-500 µg ml^{-1}) were used as a standard. In control, 0.5 ml PBS was added instead of extract. Each experiment was done in triplicate and average was taken. Nitric oxide free radical scavenging activity was calculated using the following formula:

Percentage inhibition = [(OD of control- OD of test) / OD of control] × 100

Reducing Power Activity: The reducing potential of the extract is measured when Fe^{3+} is reduced to Fe^{2+} which then reacts with ferric chloride to form a ferric-ferrous complex. Colour of the reaction solution changes from yellow to various shades of green and blue depending on the reducing power of the compound [19]. Different concentrations of ethanolic stem bark extract as well as ascorbic acid or standard (50 µg ml^{-1}, 100 µg ml^{-1}, 250 µg ml^{-1}) were dissolved in 2.5 ml of phosphate buffer (0.2 M, pH 6.6). 2.5 ml of 1% potassium ferricyanide was mixed with the above solutions and incubated at 50 °C for 20 min. After incubation, 2.5 ml of 10% TCA was added to the mixtures, followed by centrifugation at 3000 rpm for 10 min. The supernatant was collected and mixed with 1 ml distilled water and 500 µl of 0.1% ferric chloride. The absorbance of the resultant solution was measured at 700 nm. A color change from yellow to various shades of green and blue occur depending on the reducing power of the compound.

Statistical Analysis: In this study, a total of four variables (Percentage DPPH free radical inhibition, total antioxidant activity, percentage nitric oxide scavenging activity and reducing power activity) were considered in four different tests with ethanolic stem bark extract and standard (Ascorbic acid). Mean and standard error values were calculated for DPPH free radical scavenging activity, total antioxidant activity, nitric oxide scavenging activity and reducing power activity, separately for various ethanolic stem bark extract concentrations standardized for each variable. Further, the mean values for each variable were expressed as an average of three observations made and expressed as mean ± S.E. statistical significance was tested using ONE WAY ANOVA.

RESULTS:

Preliminary Phytochemical Screening: The preliminary phytochemical screening of the *K. attenuata* stem bark extract indicated the presence of various phytoconstituents as described in **Table 1**. The Physicochemical characterizations of ethanolic stem bark extract revealed that it was reddish brown with amorphous powder consistency and aromatic odor. There was 17.8% w/w yield obtained for the ethanolic stem bark extract.

Quantitative Estimation of the Extract: Quantitative estimation of *K. attenuata* stem bark extract was carried out using standard curves of gallic acid (Total phenolic content) and quercetin (Total flavonoid content). 1000 µg ml^{-1} of the extract was found to possess a significant amount of phenolic contents (387.6 µg ml^{-1} gallic acid equivalents) and flavonoid content (84.12 µg ml^{-1} quercetin equivalent).

TABLE 1: PRELIMINARY PHYTOCHEMICAL SCREENING OF THE EXTRACT

S. no.	Phytoconstituents	Tests performed	Ethanolic extract
1	Carbohydrate	Molisch test, Fehling's test, Barfoed's test, Benedict's test	+ve
2	Alkaloids	Dragendroff's test, Mayer's test, Wagner's test, Hager's test	+ve
3	Glycosides	Borntrager's test, Legal's test, Baljet test	+ve
4	Flavonoids	Alkaline reagent test, Shinoda test	+ve
5	Saponins	Foam test, Blood hemolysis test	+ve
6	Phenolics & Tannins	Ferric chloride test, Lead acetate test	+ve
7	Fixed oils & Fat	Spot test, Saponification test	- ve
8	Steroids	Libermann's Burchard test, Salkowski's test	+ve
9	Triterpenoids	Tin and thionyl chloride test	- ve
10	Gums & Mucilage	Precipitation test	- ve

***In-vitro* Antioxidant Studies:**

DPPH Free Radical Scavenging Assay: The DPPH Free radical scavenging activity was determined regarding percentage inhibition of DPPH free radical. The percentage inhibition by the ethanolic extract was found to be 54.87%, 71.09%, and 87.29% and of standard ascorbic acid was 80.67%, 85.57% and 94.90% at concentrations of 10, 50 and 100 µg ml^{-1} respectively **Fig. 1**. Therefore, the extract demonstrated an increasing percentage inhibition of DPPH free radical with an increase in concentration same as ascorbic acid indicating comparable scavenging activity against DPPH free radicals. The IC_{50} value of ethanolic stem bark extract was obtained using the linear regression equation which was estimated at 17.97 µg ml^{-1}.

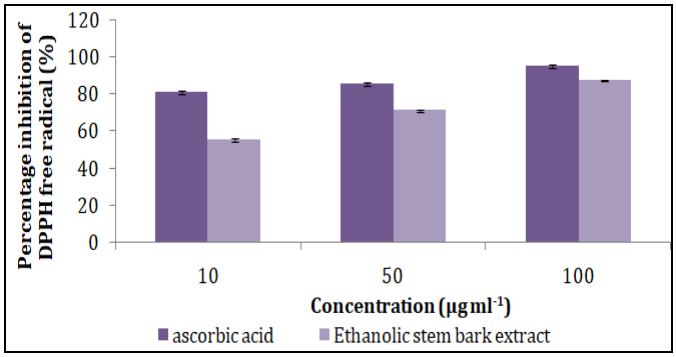

FIG. 1: COMPARISON OF DPPH RADICAL SCAVENGING ACTIVITY OF ETHANOLIC STEM BARK EXTRACT OF *K. ATTENUATA* AND ASCORBIC ACID

Total Antioxidant Activity: The incubation of extract with molybdenum (VI) led to the formation of a green phosphate/Mo (V) complex indicating the presence of antioxidant components in the extract, which was assessed by measuring at 695 nm. Total antioxidant (both water soluble and fat soluble) activity of ascorbic acid was considered as the reference standard **Fig. 2**.

A significant increase in total antioxidant activity was seen with an increase in the concentration of extract. The results of the assay can be expressed regarding ascorbic acid equivalents. 100 µg ml^{-1} of ethanolic stem bark extract of *K. attenuata* showed an absorbance of 0.5091, which is equivalent to 81.05 µg ml^{-1} of ascorbic acid indicating a comparable antioxidant potential for the extract.

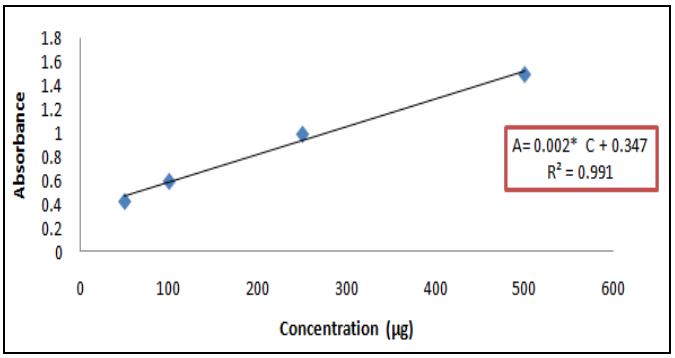

FIG. 2: TOTAL ANTIOXIDANT ACTIVITY OF STANDARD ASCORBIC ACID

Nitric Oxide (NO) Scavenging Activity: Percentage, nitric oxide scavenging activity of different concentrations of ethanolic stem bark extract, was determined and compared with that of standard (ascorbic acid). The percentage scavenging activity for NO of extract was 52.6%, 57.8%, 68.1% and 79.5% at concentration of 50, 100, 250, 500µg ml^{-1}. At the same concentration, the ascorbic acid inhibited NO and exhibited percentage NO scavenging activity as 51.5%, 57.2%, 64.4%, and 73.5% respectively.

Therefore, the best antioxidant activity regarding scavenging activity for NO (79.5%) was observed at a concentration of 500 µg ml^{-1}. Thus, ethanolic stem bark extract exhibited an efficient activity for scavenging NO comparatively higher than ascorbic acid, the standard. The IC_{50} value of both ascorbic acid and the extract was obtained using the linear

regression equation which was found to be 27.61 µg ml^{-1} and 25.52 µg ml^{-1} respectively.

Reducing Power Activity: The reducing power activity of various concentrations of ethanolic extract of stem bark of *K. attenuata* was compared with that of ascorbic acid as standard.

The reducing power activity of the extract was found to increase with the increase in concentration, and the highest absorbance of 1.33 ± 0.0871 was given by 250 µg ml^{-1} of extract, compared to an absorbance value of 1.72 ± 0.0493 for standard, ascorbic acid.

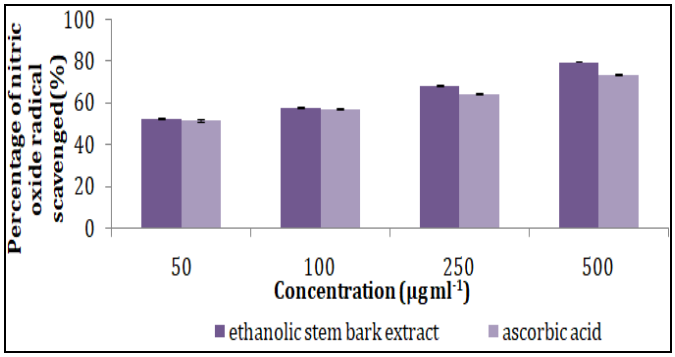

FIG. 3: COMPARISON OF NITRIC OXIDE FREE RADICAL SCAVENGING ACTIVITY (%) OF ETHANOLIC STEM BARK EXTRACT AND ASCORBIC ACID

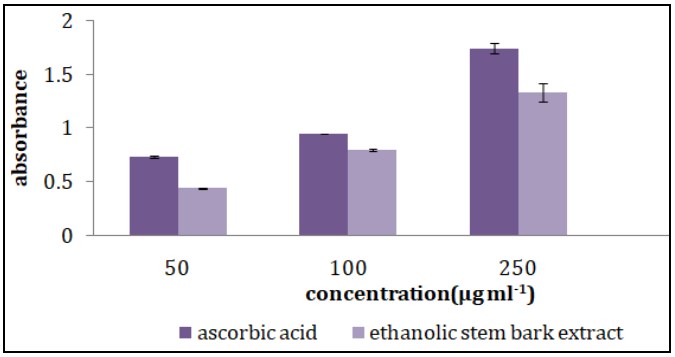

FIG. 4: COMPARISON OF REDUCING POWER ACTIVITY OF ETHANOLIC STEM BARK EXTRACT AND ASCORBIC ACID

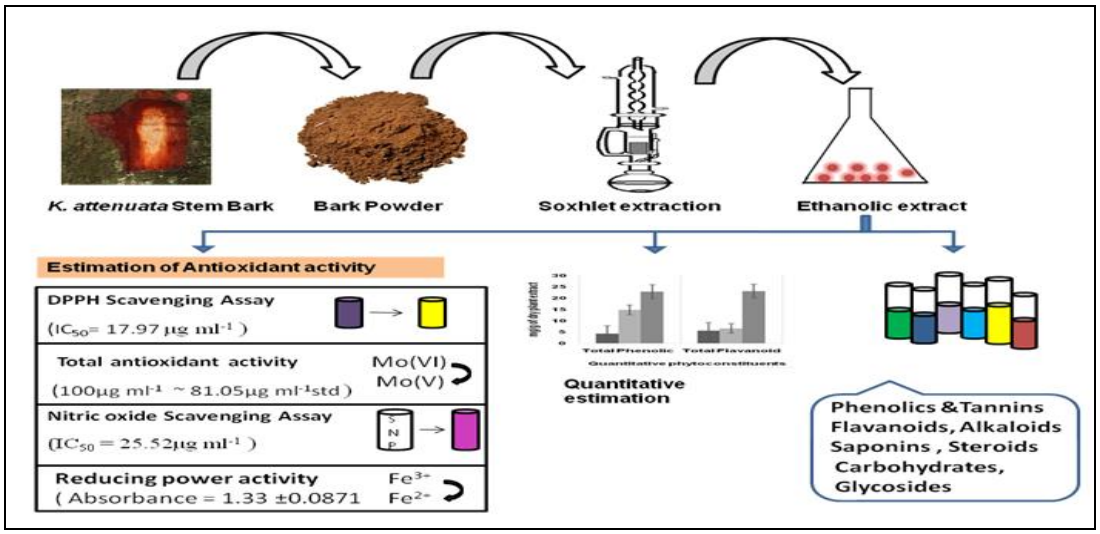

FIG. 5: THE OVERALL RESEARCH SUMMARY CAN BE REPRESENTED IN THE FIGURE

DISCUSSION: Medicinal plants have been used traditionally as folk medicines against a wide variety of disorders [20]. Various natural compounds isolated from plants have shown to possess antioxidant and radical scavenging properties which are beneficial for treating diseases caused by oxidative damage. Phenolic compounds and flavonoids are important antioxidant compounds, widely distributed in plant tissues that exhibit significant scavenging activity against free radicals [21]. In this study, the phytochemical screening of stem bark extract of *K. attenuata* confirmed the presence of phenolics in the extract. Further, quantitative analysis of phytochemicals revealed that the total amount of phenolic compounds and flavonoids present in the extract was equivalent to 387.6 µg ml^{-1} of gallic acid and 84.12 µg ml^{-1} of quercetin respectively. The antioxidant potential of the extract was estimated using four tests; DPPH free radical scavenging activity, total antioxidant activity, nitric oxide radical activity and reducing power activity. The significant antioxidant activity shown by the extract can be attributed to the phenolics present in the extract. In the following sections, a detailed discussion is provided on the antioxidant activity of the *K. attenuata* with reports from earlier scientific research attempts.

DPPH free radical scavenging activity (antiradical activity) is an important test to determine the

antioxidant potential of plant extracts, foods, *etc*. DPPH is a very stable organic free radical with deep violet color which on receiving a proton from any hydrogen donor, becomes yellow colored hydrazine (DPPH-H) with reduced absorbance. The hydrogen donating capacity of extract indicates its antioxidant potential. The assay was performed with different concentrations, and a maximum activity (87%) was observed at 500 µg/ml, with an IC_{50} value of 17.97 µg ml^{-1}. This antiradical activity may be attributed to the presence of phenolics which can act as hydrogen donors for reducing DPPH free radical [22]. A comparatively higher DPPH free radical scavenging activity had been reported in ethanolic whole plant extract of *K. angustifolia* with an effective concentration of sample required to scavenge DPPH radical by 50% as 13.90 ± 1.35 µg ml^{-1}.[23] Antioxidant activity of methanolic stem bark extract of *M. dactyloides* was determined using DPPH scavenging assay. IC_{50} of the sample was found to be 34.34 ± 0.18 µg ml^{-1}. [24] Hence, the activity was found to be lower for *M. dactyloides* while comparing the activity with *K. attenuata*, in this study.

The total antioxidant capacity of the extract can be compared with that of the standard; ascorbic acid using phosphomolybdenum method in which reduction of Mo (VI) to Mo (V) and the subsequent formation of a green phosphate / Mo (V) complex at acidic pH occurs at 695 nm. Since a phospho-molybdenum method is quantitative, it gives the antioxidant activity expressed as the number of equivalents of ascorbic acid [17]. In this study, 100 µg/ml ethanolic stem bark extract was found to be equivalent to 81.05 µg ml^{-1} of ascorbic acid which effectively reduced the free radicals. In this study, nitric oxide scavenging activity of extract demonstrated higher activity compared to ascorbic acid of same concentration (50, 100, 250 and 500 µg ml^{-1}). In the assay of nitric oxide scavenging, sodium nitroprusside decomposes in aqueous solution and releases nitric oxide which upon reaction with oxygen produces stable nitrite ions. The nitrite ions react with Griess reagent forming a pink color which was measured at 546 nm. Scavengers of nitric oxide donate protons to the free radicals causing reduced nitrite production and hence reduced absorbance which was used as a measure to find out the extent of NO radical scavenging [25]. Since, the extract caused the decrease in the amount of nitrite produced by the decomposition of sodium nitroprusside *in-vitro*; action can be attributed to direct NO scavenging which may be related to the presence of flavonoids and phenolic compounds in the extract. Nitric oxide scavenging activity of methanolic stem bark extract of *M. malabarica* with an IC_{50} of 500 µg ml^{-1} was determined [26]. Ethanolic stem bark extract of *K. attenuata* (IC_{50} = 25.52 µg ml^{-1}) exhibited higher scavenging activity for Nitric oxide compared to *M. malabarica*.

The electron donating ability of the extract which helps to stabilize the free radicals and subsequently break the free radical reaction chain indicates the reducing power of extract [27]. This was found to increase with an increase in the concentration of ethanolic stem bark extract showing comparable values with that of the standard. Phenolic compounds; flavonoids and phenolic compounds exhibited antioxidant activity through their reductive capacity in a Fe^{3+} - Fe^{2+} system indicating that their presence might be the reason behind the reducing capacity of extracts [28]. These results support the antioxidant activity of the extract. Reducing power activity of different extracts of aril and kernel of *K. attenuata* have been reported previously [29].

Ethanolic stem bark extract of *K. attenuata* showed promising *in-vitro* antioxidant activity (phenolics and flavonoids being the major contributors) making it a potential source for natural antioxidants which can protect from free radical-induced oxidative stress and following disorders like hepatotoxicity, cancer, inflammation, *etc*. systematic screening and characterization of active principles are recommended so that investigation of other pharmacological activities like hepato-protective activity, anticancer activity *etc*. on ethanolic stem bark extract of *K. attenuata* can be carried out.

CONCLUSION: The present study on ethanolic stem bark extract of *K. attenuata* which contains phenolic and flavonoid compounds showed promising antioxidant activity. Further, a detailed study is recommended to determine the active constituents present in the extract, so that researches on other pharmacological activities of stem bark of *K. attenuata* can be conducted.

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CONFLICT OF INTEREST: The authors declare no conflicts of interest in preparing this article.

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