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Original Article

Comparative Assessment of the Anti-oxidant and Anti-clastogenic Activity of *Morus alba* Leaves

Anjali Raj[1*], Kenganora Mruthunjaya[2], Subba Rao Venkata Madhunapantula[3] and Santhepete Nanjundaiah Manjula[1*]

[1]Department of Pharmacology, JSS College of Pharmacy, JSS University, Mysuru, Karnataka, INDIA.
[2]Department of Pharmacognosy, JSS College of Pharmacy, JSS University, Mysuru, Karnataka, INDIA.
[3]Department of Biochemistry, Center of Excellence in Molecular Biology and Regenerative Medicine (CEMR), JSS Medical College, Mysuru, Karnataka, INDIA.

ABSTRACT

Objective: The present study was conducted to investigate the anti-oxidant and anti-clastogenic activity of fractions extracted from *Morusalba* (Moraceae) leaves. **Methods:** The leaves of *Morus alba* were sequentially extracted using n-Hexane, Petroleum Ether, Ethyl Acetate, Methanol and Water, and Total phenolic content was determined using Folin-Ciocalteau (FC) reagent. The anti-oxidant property of each fraction was determined using 2-Deoxy-D-ribose, 2, 2-Diphenyl-1-picryl-hydrazyl (DPPH) and Hydroxyl radical scavenging assay. Furthermore, anti-clastogenic potential of these fractions was determined using calf-thymus DNA in the presence of Fenton's reagent followed by analyzing the samples by agarose gel electrophoresis. **Results:** All fractions showed the presence of varied amounts of total phenols and exhibited anti-oxidant activity as determined by scavenging the corresponding radicals. Among these fractions the methanol fraction (MEMA) showed the maximum phenol content as well as anti-oxidant activity. In addition MEMA could inhibit the breakage of DNA strands (anti-clastogenic activity) compared to other fractions tested. **Discussion:** Results of this study suggests that MEMA is likely to possess better anti-oxidant and anti-clastogenic activity.

Key words: *Morus alba*, MEMA, Hydroxyl, Phenols, Fenton reaction.

Correspondence :

Dr. S.N. Manjula,
Asst. Professor and Head,
Department of Pharmacology,
JSS College of Pharmacy, JSS University,
Mysuru-570015, INDIA.

E-mail: snmanjula@jssuni.edu.in
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INTRODUCTION

Morus alba Linn, commonly known as white mulberry and as Tut in India, belongs to the family Moraceae. Commercially white mulberry is cultivated throughout the world to raise silkworms.[1] However, the fruits have been used widely to treat various diseases such as diabetes mellitus, cardiovascular health, cancer and cognition. Prior studies have shown that the fruit extract of *Morus alba* induces nerve growth factor release in rat hippocampus[2] and help in the treatment of anxiety conditions.[3] Moracin, the major constituent of the plant especially the leaf part, has been shown to inhibit PDE_4suggesting its role in cognition and memory.[4] Though the fruits are delicious and edible, the anti-oxidant levels are found to be much higher in the leaves.[5] The major constituents of plant leaves include Chalcomoracin, Morachalcone A-C and flavanoids such as Luteolin, Rutin and Chlorogenic acid.[6-8] Hence, the present study is aimed at screening of extracts prepared using solvents with increasing polarity from the*Morus alba* leaves for their potential to inhibit free radicals (anti-oxidant activity) as well as to protect the DNA from free radical induced breaks (anti-clastogenic activity).

MATERIALS AND METHODS

Plant material

Morus alba plants were procured from Srirangapatna, Mysuru, Karnataka and was authenticated by a botanist. The leaves were separated,washed with tap water and dried under shade. The pulverization of leaves was carried out by a mechanical grinder and then subjected to extraction.

Chemicals

2-Deoxy-D-ribose, 2, 2-Diphenyl-1-picryl-hydrazyl (DPPH), Ascorbic acid, calf thymus DNA waspurchased from Sigma Aldrich Chemical Co, (St. Louis, MO, USA).Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), EDTA, acetone, ferrous sulphate ($FeSO_4$), ferric chloride, Folin-Ciocalteau reagent and Hydrogen peroxide (H_2O_2) were obtained fromMerck Millipore (Darmstadt, Germany). All other reagents used in this study were of analytical grade.

Fractionation of the Morus alba Leaves

Exhaustive Soxhlet method was used for extraction of *Morus alba* leaves. Coarse powder of the leaves was loaded into the thimble made of Whatman filter paper in the Soxhlet apparatus and successively extracted with the solvents in the increasing order of polarity viz; n-hexane, petroleum ether, ethyl acetate, methanol and finally with water. 500 ml of each solvent was used for the extraction. Each of the extract i.e. Hexane, (HEMA), Pet. Ether (PEMA), Ethyl Acetate (ETMA, Methanol (MEMA), Water (AQMA) respectively were filtered, concentrated using Flash Rotary evaporator, dried and stored in a vacuum desiccator at room temperature till use.

Determination of Total Phenolic acid Content (TPC)

Total phenolic acid content in the fractions was determined according to Mruthunjaya, K (2008)[9] with slight modification. An aliquot of the extract was mixed with 2 ml Folin-Ciocalteau reagent (previously diluted with water 1:10 v/v) and 2 ml (75 g/l) of sodium carbonate. The tubes were vortexed for 15sec and allowed to stand for 20 min at 25°C. The developed color was read by measuring the absorbance at 760 nm using UV-V is spectrophotometer (Shimadzu, USA). Total phenolic content was expressed ingallic acid equivalent, GAE.

In-vitro Anti-oxidant Assays

DPPH Radical Scavenging Assay (DRSA)

Free radical scavenging ability of the extracts was tested by DPPH Radical Scavenging Assay (DRSA) as described by Choi *et a1* (2000)[10] and Desmarchelier *et al* (1997).[11] A solution of 0.1 mM DPPH in methanol was prepared and 2.4 ml of this solution was mixed with 1.6 ml of extracts at different concentrations (20, 40, 60, 80 and 100 µg/ml).

The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 minutes. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid (2, 4, 6, 8 and 10 µg/ml) was used as reference. The experiments were carried out in triplicates and the percentage DPPH radical scavenging activity (% DRSA) was calculated by the following equation,

$$(\%DRSA)= \{(Ao–A1)/Ao\} \times 100$$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the fractions/standard.

The % of inhibition was plotted against concentration, and from the graph IC_{50} was calculated.

Hydroxyl Radical Scavenging Activity (HRSA)

Hydroxyl radical scavenging activity (HRSA) of the fractions was determined by the method of Harsh Ramakrishnan *et al*[12] with a slight modification. The reaction mixture containing 0.2 ml of 20 mM phosphate buffer (pH-7.4), 0.2 ml $FeCl_3$ (10 mM), 0.1 ml ascorbic acid (0.1 mM), 0.1 ml EDTA (1 mM), 0.1 ml H_2O_2 (10 mM), 0.2 ml of 2-deoxy-*d*-ribose (10 mM) and different (200, 400, 600, 800 and 1000 µg/ml) concentration of the fractions (1 ml each) were incubated at room temperature for 60 min. Next, 1 ml each of 1% Thiobarbituric acid and 2.8% Trichloroacetic acid were added and the final mixture kept in a boiling water bath for 30 min. The damage imposed due to free radicals was determined colorimetrically by measuring the thiobarbituric acid reactive substances (TBARS) at 532 nm. Ascorbic acid (20, 40, 60, 80 and 100 µg/ml) was used as the standard. All experiments were carried out in triplicate and average values with standard deviation represented. Percentage hydroxyl radical scavenging (% HRSA) activity was calculated using the following formula,

$$\%HRSA= \{(Ao–A1)/Ao\} \times 100$$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the fractions/standard.

The percentage inhibition was plotted against concentration to determine the $IC_{50\ value}$.

Anti-clastogenic activity of fractions on calf thymus DNA

Fenton reaction-induced calf thymus DNA protection studies were carried out according to Tsuching *et al* (2007)[13] with modifications. In brief, 30 µl of all extracts (1 mg/ml) were incubated with 10 µl calf thymus DNA (1 mg/ml), $FeSO_4$ (80 mM), H_2O_2(150 mM) and EDTA (15 mM). After 20 minutes the reaction mixture was loaded into 1% agarose gel. The electrophoresis was carried out by running the samples at 60 Volts for 90 minutes. The DNA bands were visualized by viewing the gel in a UV-trans-illuminator operating at 312 nm.

RESULTS

Determination of Total Phenolics Content

To check and identify the fraction rich in total phenols, a simple and widely used Folin Ciocalteau method was used. Gallic acid was used as a positive control as well as to prepare the standard graph. Analysis of the data (Table 1) showed a significantly high 9.54% total phenol content in MEMA compared to other fractions. Among the five fractions only HEMA had low total phenol content (5.4%). Other fractions PEMA, ETMA and AQMA had a total phenolic acid content of 6.6%, 7.19% and 9.15% respectively (Table 1).

DPPH radical scavenging ability of Morus alba fractions

DPPH free radical is a stable radical widely used for measuring the radical scavenging ability of phyto-constituents. Experimentally, DPPH reaction mixture was incubated with increasing concentrations of *Morus alba* leaf extracts and percentage inhibition compared to no-treatment group

Table 1: Total phenolic content of all the fractions of *Morus alba* leaves.

Fraction	Total Phenolics Content (mg Gallic acid/g of extract)
HEMA	54.2 ± 0.784
PEMA	66.1 ± 0.563
ETMA	71.9 ± 0.68
MEMA	95.4 ± 0.53
AQMA	91.5 ± 0.75

All values are expressed as Mean ± SEM, n=3.

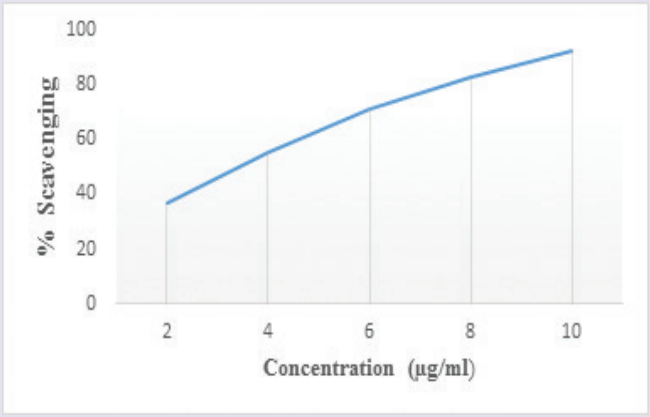
calculated. Furthermore, the IC_{50} value was also calculated for each fraction by subjecting the percentage scavenging data to excel analysis. The data showed a dose dependent increase in the scavenging activity of all the fractions of *Morus alba.* Relative comparison among these fractions showed better DPPH radical scavenging ability of MEMA with an IC_{50} of 40.89 ± 2.65 µg/ml. Other fractions HEMA, PEMA, ETMA, and AQMA exhibited an IC_{50} of 90 ± 3.05, 80.49 ± 2.15, 75.44 ± 2.32, and 63 ± 1.67 µg/ml respectively. The IC_{50} of reference standard Ascorbic acid was 3.45 ± 2.85 µg/ml (Graph 1).

Hydroxyl radical scavenging potential of Morus alba fractions

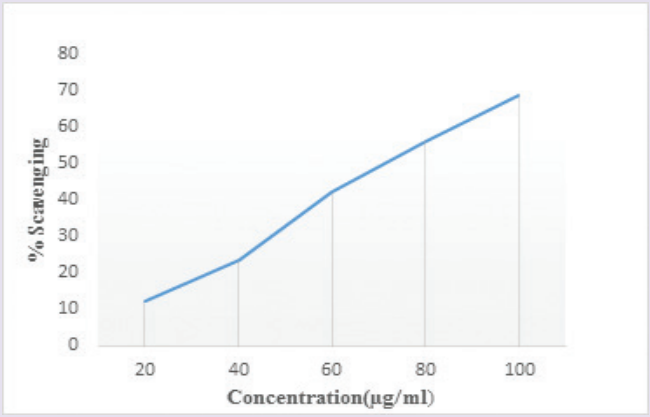
The hydroxyl radical (˙OH), the most reactive species of 'activated oxygen', is formed by successive monovalent reduction of dioxygen (O_2) in cell metabolism and is responsible for the cytotoxic effects of oxygen in organisms. The hydroxyl radicals can attack polysaccharides, proteins and nucleic acids thereby modulate their expression and/or activity in response to a variety of stress conditions. In addition, hydroxyl radicals have been shown to be involved in numerous disorders that include inflammations and cancer.[14] Therefore, detoxifying hydroxyl radicals is very important to prevent the progression of cancers in to advanced stages. Hence, in this study we have screened the *Morus alba* fractions for the ability to inhibit the production of hydroxyl radicals. Analysis of the data represented in Graph 5 showed a significant hydroxyl radical scavenging effect with MEMA with an IC_{50} value of 250.05 ± 3.34 µg/ml. Other fractions ETMA (IC_{50}-374.15 ± 3.75 µg/ml) and AQMA (IC_{50}-311.04 ± 3.67 µg/ml) also showed potent scavenging activities compared to HEMA (IC_{50}-575.37 ± 2.87 µg/ml) and PEMA (IC_{50}-548.89 ± 3.95 µg/ml). However, none of these extracts are comparable with the standard compound ascorbic acid for scavenging the hydroxyl radicals. The IC_{50} of ascorbic acid was 64.67 ± 3.43 µg/ml (Graph 4).

Effect of fractions on Fenton reaction induced clastogen

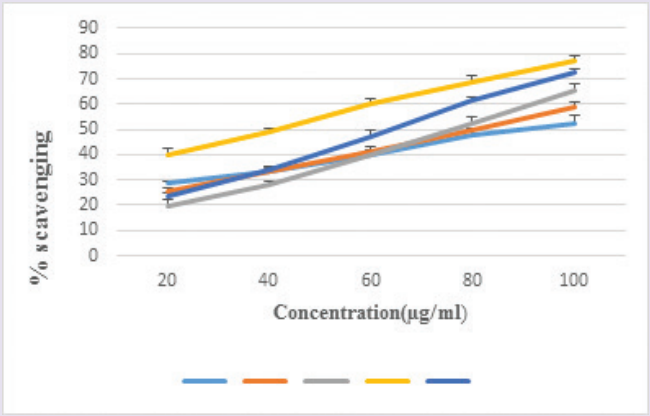
The DNA damage induced by Fenton's reaction in calf thymus DNA was used to assess the anti-clastogenic effect of *Morus alba* fractions. Fenton's reaction generates ·OH radicals, which attack intact DNA thereby generate DNA fragments.[14] The generated DNA fragments can be analyzed using agarose gel to determine the extent of degradation. Using this methodology, the efficacy of *Morus alba* fractions for protecting the Fenton reaction induced DNA damage was assessed and the results represented in Figure 1. As shown, MEMA offered the best protection (Lane #2) compared to other fractions (Lanes 3-6). Incubation with MEMA protected the DNA (Compare lane #2 with lane #7, which represent the DNA without treatment). However, incubation with other fractions failed to protect the DNA as the DNA showed a smeary pattern with more amount of medium sized and low molecular weight fragments (Compare lanes 3-6 with lane #7) similar to control DNA treated with Fenton's reagent (Lane #1).



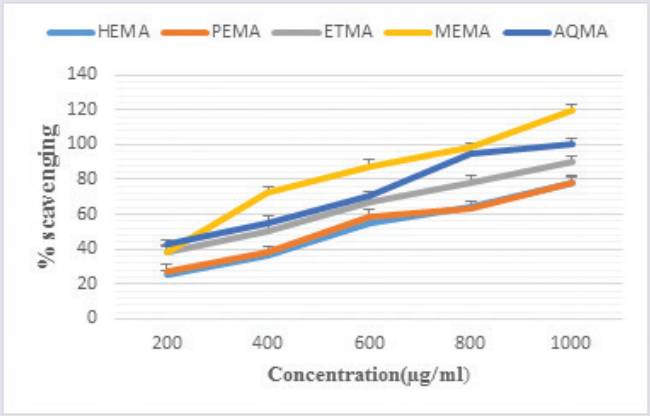
Graph 1: DPPH free radical scavenging activity of Ascorbic acid.



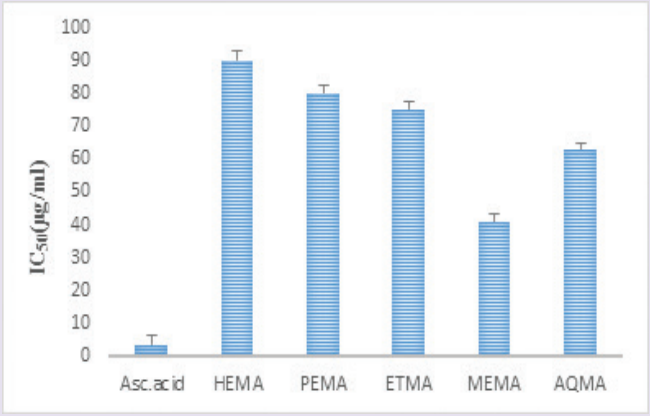
Graph 4: Hydroxyl radical scavenging activity of Ascorbic acid.



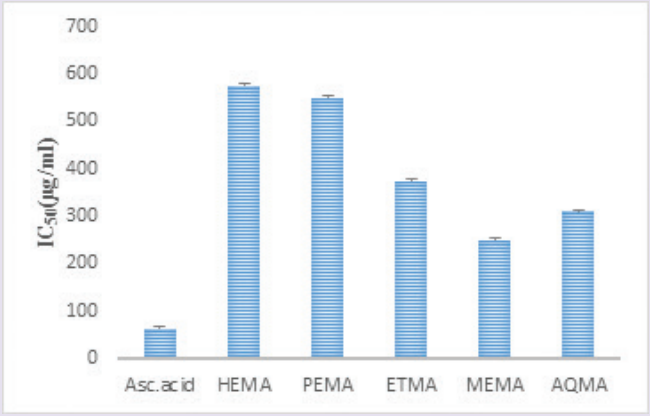
Graph 2: DPPH free radical scavenging activity of fraction of the leaves.



Graph 5: DPPH free radical scavenging activity of fractions of leaves.



Graph 3: IC_{50} values of ascorbic acid and all fractions of *Morus alba* leaves. All values are expressed as Mean ± SEM, n=3.



Graph 6: IC_{50} values of ascorbic acid and all fractions of *Morus alba* leaves for Hydroxyl radical Scavenging.
All values are expressed as Mean ± SEM, n=3.

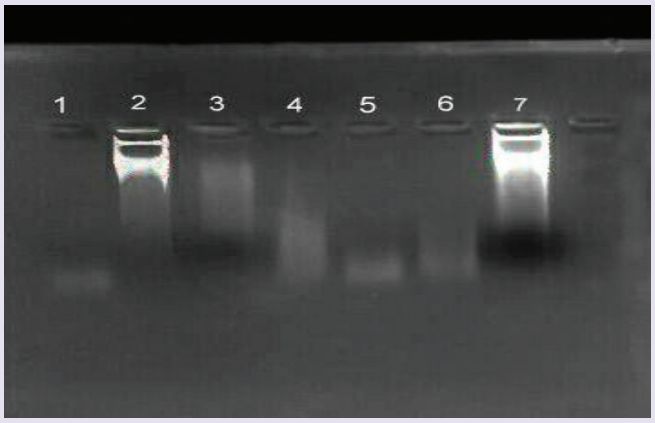


Figure 1: Anti-clastogenic activity of *Morus alba* leaves against Fenton reaction induced DNA damage in calf thymus DNA.
Lane#1: Fenton's reaction mixture (H_2O_2+$FeSO_4$+EDTA); Lane #2: MEMA+Fenton's reagent, Lane #3: ETMA+Fenton's reaction; Lane #4: AQMA+Fenton's reagent mixture; Lane #5: PEMA+Fenton's reaction, Lane #6: HEMA+Fenton's reaction and Lane #7: Calf thymus DNA; Lane #8: Blank.

DISCUSSION

Deregulated production of reactive oxygen species and free radicals resulting from oxidative stress has been implicated in the origin of various disorders including cancers, neurodegenerative diseases. Controlling the levels of free radicals/ROS by promoting the antioxidant enzymes, or by increasing the concentration of cellular reducing agents helps to reduce oxidative stress induced diseases.[15] Several studies have explored the possibility of using natural products and extracts for mitigating the levels of ROS. One such plant that has been studies for its ability to reduce oxidative stress is *Morus alba*. The roots and fruits of *M. alba* have been traditionally used for treating hypertension, rheumatism, beri-beri, pyrexia[16] etc. However, not much is known about the use of *M. alba* leaves for inhibiting ROS and ROS-induced DNA damage. Therefore, in this study we have isolated phytochemicals from *M.alba* by sequential extraction with n-Hexane, Petroleum ether, methanol, ethyl acetate and water and characterized these extracts for percentage total phenol, DPPH and hydroxyl radical scavenging activities. Next, the ability of these extracts for down-regulating free radical induced DNA damage was studied using Fenton's reaction approach.

Among the *M. alba* extracts, the MEMA exhibited the most potent DPPH radical clearing potential. DPPH is a widely used stable radical for determining the free radical scavenging activity. The assay depends on the ability of an anti-oxidant to donate hydrogen ions to DPPH radical. Since MEMA but not other extracts showed better DPPH radical scavenging activity, it is likely that it might be having more reducing agents. Characterization of these reducing agents requires additional studies. Like DPPH radical scavenging ability, the potency of *M. alba*extracts for destroying hydroxyl radicals also varied among different fractions of *M. alba*. In cells, hydroxyl radicals aregenerated by the action of Superoxide dismutase (SOD) activity on superoxide anions. The hydroxyl radicals thus produced interact with DNA thereby (a) modify purine and pyrimidine bases; (b) trigger oligonucleotide strand breaks; (c) disturb DNA-protein cross-links; and (d) cause abasic sites.[17] Since presence of unusually high levels of these radicals is highly deleterious to the cells, it is important to keep the concentration of these radicals as low as possible either by stimulating the expression of antioxidant enzymes are by externally supplying the antioxidants to cells.[15] Although many synthetic and natural antioxidant chemicals have been synthesized and tested for efficacy using *in vitro* and *in vivo* models, no universal antioxidant that can take care of oxidative stress is available. Hence, the search for potent antioxidants has been an intense area of investigation. Priorstudies on *Morus alba* have indicated the presence of potent free radical quenchers such as polyphenols, flavonoids and procyanthidins in the roots.[1] However, not much is known about the antioxidant ability of leaf extracts. Therefore, in the present study the *M. alba* leaves were extracted with n-hexane, petroleum ether, methanol, ethyl acetate and water in a sequential manner and tested the isolated fractions for antioxidant and anti-clastogenic activity. The methanol fraction of leaves showed the best scavenging activity as evidenced by better efficacy in DPPH (40.89 ± 2.65) and Hydroxyl (250.05 ± 3.34) scavenging assays, which could be due to the better extractability of methanol for extracting phenols. Since the extractability of phenolic compounds depends on the solubility, which in turn depend on the polarity of the solvent, methanol being the better polar solvent compared to n-hexane, could extract more phenolic compounds[18] and exhibit potent antioxidant and anticlastogenic activity.

The hydroxyl radicals are mutagenic andclastogenic in nature.[19] In order to test the efficacy of *M. alba* fractions against these radicals, aFenton's reaction that generates hydroxyl radicals by the reaction between H_2O_2 with ferrous sulphate was used to induce the damage of calf thymus DNA. Calf thymus DNA, extracted from the thymus glands of calves, is one of the most widely used DNA preparations in anti-clastogenic studies.[20] Even though all the fractions tested for anti-clastogenic ability showed a protective effect, MEMA exhibited the most protective effect as indicated by the presence of intact DNA that is comparable to that of normal DNA. It is predicted that the MEMA fraction, due to its high hydroxyl radical scavenging ability, might have actively scavenged the Fenton generated hydroxyl radicals and protected the DNA from the damage. However, it is not fully known, which molecule of the MEMA is responsible for this potent anti-clastogenic activity; and whether the MEMA fraction exhibit similar protective property when tested in cells and animals. Further studies are required to isolate and characterize the active molecule(s) from MEMA.

CONCLUSION

In summary, our findings indicate that the leaves of *Morus alba* possess considerable anti-oxidant activity, preferentially extracted by methanol. The methanol soluble antioxidants couldinhibit the generation of free radicals and protect the DNA from the damage induced by a clastogen. Therefore, MEMA is a potential candidate fraction for further studies aiming at isolation and characterization of the active ingredient and exploring the possibility of testing it for protecting the neurons from oxidative stress induced damage in animal model.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

ABBREVIATION USED

PDE4: Phosphodiesterase enzyme, **DNA:** Deoxy ribonucleic acid, **EDTA:** Ethylene Di-amine Tetra accetate, **DPPH-2:** Diphenyl-1-picryl-hydrazyl, **ROS:** Reactive Oxygen Species.

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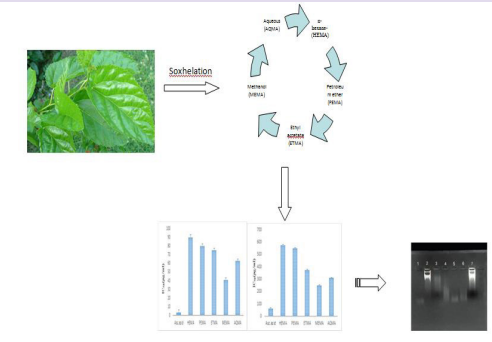
PICTORIAL ABSTRACT



SUMMARY

- All the fractions of *Morus alba* leaves were found to contain Phenolics.
- The content was found to increase with the polarity of the solvents with methanol fraction (MEMA) found to express the maximum phenols.
- The IC_{50} values of all the fractions obtained from both DPPH and hydroxyl radical scavenging assays were in match to their phenolic contents. MEMA depicted the minimum IC_{50} values for both the assays indicating the significance of phenols in anti-oxidant activity.
- The clastogenic effect generated by Fenton's reaction was almost completely reversed by the MEMA fraction. The ethyl acetate and aqueous fractions (ETMA & AQMA) also exhibited a slight protective effect to the clastogen.
- The results indicate the good hydroxyl scavenging activity (250.05 ± 3.34) of MEMA fraction of *Morus alba* leaves qualifying it to be a good neuroprotective agent which requires further *in vitro* and *in vivo* studies for substantiation.

ABOUT AUTHORS

Dr. S.N. Manjula, Ph.D.: Completed M. Pharm, in Pharmacology in the year 2001 from Govt. College of Pharmacy, Bangalore. She was awarded with Ph.D. in 2010 from Manipal University, Manipal. Presently Dr. S.N. Manjula is working as an Assistant professor and Head, Dept. of Pharmacology at JSS College of Pharmacy, Mysore. She has been working here since 2001. Her primary area of research is cancer and other areas of research are inflammation, chemoprevention, chemoprotection and diabetes. Currently she is involved in screening of novel EGFR inhibitors and several herbs for their *in-vitro* and *in-vivo* antitumor activity.

Dr. K. Mruthyunjaya, Ph.D.: Completed M. Pharm, in Pharmacognosy in the year 1999 from Govt. College of Pharmacy, Bangalore and PhD 2009 from Rajiv Gandhi University of Health Sciences, Karnataka, Bengaluru. Presently Dr. Mruthunjaya is working as an Assistant Professor in the Dept. of Pharmacognosy at JSS College of Pharmacy, Mysuru, which is a constituent college of JSS University, Mysuru, India. He has long 17 years of teaching and research experience in the said college. His area of research is identifying, screening and validation of antioxidant herbs for their various biological properties.

Dr. SubbaRao V. Madhunapantula, Ph.D.: Received Ph. D. in Biochemistry from University of Mysore. Subsequently Dr. Madhunapantula underwent post-doctoral training in the department of Biochemistry and Molecular Biology between 2002 – 2004 and in the department of Pharmacology between 2004 – 2009 at Penn State College of Medicine, Hershey, Pennsylvania, USA. Currently Dr. Madhunapantula is a Professor in the Center of Excellence in Molecular Biology and Regenerative Medicine, JSS Medical College, JSS University, Mysuru.

Anjali Raj: Currently working as a research scholar in the Dept. of Pharmacology, JSS College of Pharmacy, Mysuru has obtained her M.Pharm from the same institute. Mainly interested in neurodegenerative diseases, she is presently working on combination therapy for mitigating dementia effects in Alzheimer's disease.