International Journal of Biology Research
ISSN: 2455-6548
Impact Factor: RJIF 5.22
www.biologyjournal.in
Volume 2; Issue 4; October 2017; Page No. 12-16

Exploration of the phytoconstituents and potentials of the *Mesua ferrea* collected from the Assam region in India for antioxidant and microbicidal activity

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Abstract
Free radicals are the naturally formed molecules in the body which possess several roles. Under stress conditions, their production may increase which may cause deleterious effects. Free radicals are scavenged efficiently by plant products which are having various type of phytochemicals including phenolic compounds and flavonoids. The present study focused on the quantification of the total phenolic and flavonoid content in the methanolic extract of the *Mesua ferrea* and determination of its antioxidant potentials and antimicrobial activity. The study showed that plant extract was having a significant amount of phenolic and flavonoid content. The extract was efficiently scavenging the DPPH radicals and showed a considerable total antioxidant and ferric reducing power. For the antimicrobial study, gram-positive as well as gram-negative bacteria were used, and the extract was showing the ability to inhibit their growth. Thus, *M. ferrea* may be a potential candidate as a future antioxidant and antimicrobial agent.

Keywords: *Mesua ferrea*, antioxidant, antimicrobial, free radicals, plant extract

Introduction
A condition described as oxidative stress arises when free radicals conquer the body's capacity to control them. Free radicals are atoms or group of atoms competent of independent existence containing an unpaired electron in outermost orbital which is unstable and extremely reactive. In cells, free radicals are formed as a result of enzymatic reactions such as those involved in prostaglandin synthesis, phagocytosis, respiratory chain, arachidonate pathways and in cytochrome P-450 system; likewise in non-enzymatic reactions such as those reactions of oxygen with organic compounds. Generation of free radicals from external environment includes exposure to ozone, X-rays, cigarette smoking, industrial chemicals and air pollutants [1]. The free radicals or ROS in cells are capable of damaging biological macromolecules like lipids, carbohydrates, DNA and proteins leading to cell disruption and homeostatic damage [2]. ROS generated oxidative stress is linked to the occurrence of chronic diseases such as osteoporosis, cancer and cardiovascular disease [3]. ROS have also been associated with the initiation and cause of age-linked eye diseases, diabetes mellitus, and neurodegenerative diseases like Parkinson's disease. Oxidative stress is considered to be a significant contributor to many inflammatory conditions, acquired immunodeficiency syndrome, hemochromatosis, hypertension, gastric ulcers, emphysema and various neurological disorders, and many others [4, 5]. In our body, cells are defended against oxidative stress through an interconnecting network of antioxidant enzymes. Antioxidants are molecules that hinder/quench free radical chain reactions or neutralize them by donating an electron, thus inhibiting cellular damages. Antioxidants act as a radical scavenger, peroxide decomposer, a hydrogen donor, singlet oxygen quencher, electron donor, enzyme inhibitor synergist and metal-chelating agents [6]. Based on their activity antioxidants are categorized as enzymatic and non-enzymatic. *In vivo* antioxidants, i.e., the enzymes that act as a defense against free radicals are superoxide dismutase, catalase, glutathione peroxidase. Non-enzymatic antioxidants include vitamin C (Ascorbic acid), vitamin E (tocopherols), glutathione, carotenoids, and beta-carotene. These micronutrients are not produced in our body, so they must be supplied in the diet [4]. Antioxidants counteracts to protect across a variety of diseases such as aging, algesia, arthritis, atherosclerosis, asthma, autoimmune diseases, cataract, diabetes mellitus, eczema, cancer, inflammatory diseases, genetic disorders and much more [7]. Due to the increasing risk of a large number of deadly human diseases, there has been a global need toward the use of natural substances as antioxidants obtained from medicinal plants. Studies have proved the presence of various compounds in medicinal plants that act as free radical scavengers [8-10]. Natural antioxidants would be a promising

alternative against synthetic antioxidants due to their lower cost and no harmful effects in food, cosmetic and therapeutic industries [11]. There has been an increase in morbidity and mortality rates due to the growing number of antibiotic-resistant bacterial strains of clinically significant pathogens, leading to the emergence of multi-drug resistant strains. The limitation of use due to high cost, and non-availability of new generation antibiotics have led to the search for antimicrobial agents of plant origin with more potent and effective compounds for synthesizing new antimicrobial drugs.

In this study, the antioxidant activity and antimicrobial property of *Mesua ferrea* have beenevaluated. *M. ferrea* commonly known as *Ceylon ironwood* belongs to the family Guttiferae, is a medium to large size evergreen tree with short trunk attaining a height of 18 to 36m. It is native to tropical Southeast Asian countries like India, Burma, Thailand, Cambodia, Malaysia, Myanmar, Singapore, Sri Lanka and Vietnam [12]. It is distributed in India in the mountains of the eastern Himalayas and East Bengal and the North East including Assam [13]. Different parts of the plant are used as traditional medicine in India, China, Thailand, and Malaysia. The Tribals of Assam value this plant for its advantageous properties like antiseptic, tonic, worm control, purgative and blood purifier [14]. Decoction or infusion of roots and barks are used for the treatment of gastritis and bronchitis. In traditional Thai medicine it is used for the treatment of cold, fever, asthma, as well as carminative, cardiotonic, expectorant, antipyretic and diuretic agent [12]. Dried powdered flowers along with butter or ghee are given in the treatment of bleeding piles as well as dysentery with mucus [15]. *M. ferrea* has varied use in Ayurveda, Siddha, and Unani. In Ayurveda, it is used for the treatment of inflammation pain, uterine bleeding and rheumatoid conditions [16, 17]. In the Unani system, it is used for a variety of recipes such as, "Jawarish Shehryaran," a liver and stomach tonic, "Hab Pachaluna," as an appetizer, etc. [12, 18]. Previous studies have shown that the extract of this plant has anti-inflammatory, anti-venom, antispasmodic and anticancer activities [17, 19-21].

Based upon the widespread use of this plant in Ayurvedic medicine, Unani medicine and ethnomedicine, the phytochemical analysis, antioxidant activity and antimicrobial activity of *M. ferrea* was carried out. The antioxidant activity was determined by DPPH scavenging assay, total antioxidant assay, and ferric reducing assay. The total phenol content and total flavonoids content were also determined quantitatively. The antimicrobial activity was determined against *Escherichia coli*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Salmonella enterica* and *Pseudomonas putida* by the agar gel diffusion method.

Material and Method

Chemicals required

2, 2 diphenyl 1-picrylhydrazyl (DPPH), Aluminum chloride ($AlCl_3$), Ammonium molybdate, Ascorbic acid, Ferric chloride ($FeCl_3$), Folin Coicateau reagent, Gallic acid, Methanol, Phosphate buffer, Potassium acetate, Potassium ferricyanide, Quercetin, Sodium carbonate (Na_2CO_3), Sodium dihydrogen phosphate (NaH_2PO_4), Sulfuric acid (H_2SO_4), Trichloroacetic acid (TCA).

Sample Collection and Extract Preparation

Leaves of *Mesua ferrea* were collected from Darrang district of Assam in the month of December 2016. The leaves were washed thoroughly and dried under the shed. The dried leaves were crushed and milled to a fine powder with a mixer grinder. 20g of leaf powder was measured, and the extract was prepared using Soxhlet apparatus taking 90% methanol as solvent. The device was run for 48 hours, and then the extract was concentrated to dryness using Rota-vapor maintaining a temperature of 50°C. The samples were stored in the refrigerator for further experimentation.

Total Flavonoid Content

The flavonoid content of *M. ferrea* was determined according to Aluminium Chloride reducing method [22]. Plant extract (0.2 ml of 200 µg/ml) was prepared in 0.6 ml methanol. Aluminium chloride (40 µl, 10 %), Potassium acetate (40 µl, 1 M) and distilled water (1120 µl) was added in the mentioned order and incubated for 30 minutes at room temperature. Absorbance was taken at 420 nm using ELICO SL 210 UV VIS spectrophotometer. Quercetin was used as the standard compound.

Total Phenol Content

Folin Coilcateau method was used to measure the total phenolic content of *M. ferrea* [23]. Plant extract (500µl of 1mg/ml) was mixed with 2 % Na_2CO_3 (2ml), 10 % Folin Coicateau reagent (2.5 ml) and vortexed. The reaction mixture was incubated for 15 minutes at 45^0C in a water bath. Absorbance was taken at 765 nm. Gallic acid was used as the standard compound.

DPPH Scavenging Activity

The *in vitro* antioxidant property of the 90% methanolic extract of leaves of *M. ferrea* was evaluated by 2, 2 diphenyl 1-picrylhydrazyl (DPPH) free radical scavenging activity [24]. 0.135mM DPPH was dissolved in 100% methanol (4mg DPPH in 75ml methanol). Plant extract ranging from concentration 0.9-9 of 100 µg/ml was prepared. 1ml of 0.135mM DPPH and 1ml of plant extract of different concentrations were mixed in test tubes and incubated in the dark for 30 minutes at room temperature. After incubation, the absorbance of the mixture was taken at 517nm using ELICO SL 210 UV VIS spectrophotometer. Ascorbic acid was used as a standard. The DPPH scavenging activity was calculated as:

$$\%\text{DPPH scavenging activity} = \{(Abs_{control} - Abs_{sample})/Abs_{control}\} \times 100$$

Where,

$Abs_{control}$ = Absorbance of methanol + DPPH

Abs_{sample} = Absorbance of sample (plant extract/ Ascorbic acid) + DPPH

Ferric Reducing Power Assay

The reducing power of methanolic extract of *M. ferrea* was evaluated by following the method of Oyaizu [25]. Plant extract having a concentration from 40-100 of 100 µg/ml was prepared in distilled water. Plant extract (1 ml) of different concentration was mixed with phosphate buffer (1.5 ml, pH

6.6. 0.2 M) and 1% Potassium ferricyanide (1.5 ml) and incubated in water bath for 20 minutes at 50^0C, followed by addition of trichloroacetic acid (10%, 1.5ml) and centrifuged at 4000 rpm using REMI R-8C laboratory centrifuge for 20 minutes. The supernatant (1.5 ml) was collected and mixed with distilled water (1.5 ml) and Ferric chloride (0.1%. 0.3 ml). The mixture was vortexed and absorbance was measured using ELICO SL 210 UV VIS spectrophotometer at 700nm. Gallic acid was used as a standard reference compound for the study.

Total Antioxidant Activity

The total antioxidant activity of *M. ferrea* was evaluated *in vitro* according to the phosphor-molybdenum method by following the procedure of Prieto *et al.* [26]. Plant extract of different concentrations in ethanol (0.1 ml) was mixed with 1.9 ml reagent (28mM NaH_2PO_4, 4mM Ammonium molybdate, and 0.6M H_2SO_4) and incubated in water bath at 95^0C for 90 minutes. After incubation absorbance was taken at 695 nm using ELICO SL 210 UV VIS spectrophotometer. Ascorbic acid was used as a standard.

Test Microorganisms

Five bacterial strains namely *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Salmonella enetrica* and *Pseudomonas putida* were used for the antibacterial assay. The glycerol stocks of these bacterial strains were revived in nutrient broth by incubating at 37^0C for 24 hours.

Antibacterial Assay

The antibacterial property of *M. ferrea* was evaluated using agar gel diffusion method. Nutrient agar media was autoclaved and after cooling was poured onto Petri dishes. Bacterial culture (100 µl) was spread on the plates after solidification of the plates. Wells were made on the solidified media and plant extract was filled in the wells. The plant extract having concentration 5mg/ml and 10mg/ml were used. Tetracycline (30 mcg) and DMSO were used as a control.

Result and Discussion

Plants and herbs are an integral part of Ayurveda, Unani medicine and ethnomedicine. The plants have been used effectively and efficiently for the treatment of severe maladies because of the presence of several phytochemicals. These phytochemicals have been found to possess the ability to scavenge free radicals which cause several diseases in humans like cancer, atherosclerosis, Alzheimer's disease, Parkinson's disease and cardiovascular diseases [3, 27]. The antioxidant enzymes present in our body can neutralize the free radicals which are necessary for protection against bacterial and viral infection, but the excess amount of the free radicals can be lethal to our body [28]. Experimentally, the use of plants or their products has been found to enhance the antioxidative machinery of the animal system which could be helpful to scavenge the radicals generated in our body [29, 30]. Not only this, plants and their products have been found to be effective in treating or delaying the various disease [31-33]. This effect of plant products may be because of the presence of multiple phytochemicals which have been able to curb oxidative stress. Along with antioxidant activity, the plants products have also shown antimicrobial activity. The careless use of antibiotics has given rise to another serious trouble, i.e., antibiotic resistance. Phenolic compounds are one of the significant components of the plant products. They have been found to possess antimicrobial activity [34]. The plant-based compound can be a cheaper substitute for synthetic drugs that have severe side effects. The need of finding novel compounds with antioxidant and antimicrobial activity is very urgent at present. Keeping this in mind, the antioxidant and antimicrobial activity of the methanolic extract of leaves of *Mesua ferrea* were studied.

Polyphenolic Compounds

The polyphenolic compounds were quantitatively determined by total phenol content assay and total flavonoids content assay. The total phenolic content of methanolic extract of *M. ferrea* was found to be 30.97mg equivalents of gallic acid per 1g of the plant extract. The presence of phytochemicals especially phenolic compounds such as phenolic acids, flavonoids, tannins, curcuminoids, quinines, etc. are important components of radical scavenging activity and antimicrobial activity of these plants [34]. The total flavonoid content for *M. ferrea* was 45.35mg equivalents of quercetin per 1g of the crude plant extract. On comparison of our study with the previous study of Khaleel *et al.* [35], it was found that our sample which was collected from the Assam region, was having much more total phenolic and flavonoid content than the sample obtained from the Ujjain region in Madhya Pradesh in India. In another study of Udayabhanu *et al.* [36], the phenolic and flavonoid contents were higher than our sample, and the collection of the sample in their study was the Kerala region in India. So, selection of the region for the collection of the sample may be an important factor for differences in the phenolic and flavonoid content.

In one of the study, the flavonoid content was proven to possess anti-inflammatory effect and thought to protect cells against free radical damage [37]. The presence of phenolic compounds and flavonoids suggested that the plant under experimentation has antimicrobial and antioxidants activity which was further evaluated experimentally.

Antioxidant status

The antioxidant status of the plant was determined by DPPH radical scavenging activity assay, total antioxidant assay and ferric reducing assay. The amount of the antioxidant phytochemicals in plant extract is directly proportional to the DPPH scavenging ability. More the antioxidant compounds more will be the DPPH scavenging activity. The IC50 value of *M. ferrea* was found to be 6.66µg/ml which is very significant as compared to the IC50 value of reference compound ascorbic acid with IC50 value 6.62µg/ml. The DPPH scavenging activity of plant extract was almost similar to the DPPH scavenging activity of the pure compound, ascorbic acid. The reducing power of the methanolic extract was found to be equivalent to 7.2µg of gallic acid per 100µg o the plant extract. The total antioxidant activity of *M. ferrea* methanolic extract was equivalent to 15.42µg of ascorbic acid per 25µg of plant extract. The higher antioxidant activity corroborates the DPPH scavenging activity and ferric reducing power of the plant extract.

Antimicrobial Activity

The antimicrobial activity was determined by agar gel diffusion method against both pathogenic and non - pathogenic bacterial strains. The methanolic extract of *M. ferrea* was able to inhibit the growth of all the five test bacteria. *M. ferrea* (10mg/ml) formed the zone of inhibition having diameter 14mm, 13mm, 19mm, 18mm and 17 mm for *E. coli, S. aureus, P. puitda, S. enterica and S. pyogenes*, respectively. It inhibited the growth of both gram-positive and gram-negative bacteria. The plant shows a promising source of potential antimicrobial compounds.

Conclusion

Plants are a rich source of phytochemicals with antimicrobial and antioxidant activity. In our study also, this fact has been supported where the methanolic extract of *Mesua ferrea* showed significant antimicrobial and antioxidant activity. The phenolic compounds and flavonoids which were quantitatively determined were responsible for these pharmacological properties of the plant. The study also bolsters the therapeutic potential of the plant in ethnomedicine and Ayurveda. Further studies need to be done to determine the other pharmacological properties of the plant.

Acknowledgement

Financial support from Lovely Professional University in the form of M.Sc. dissertation Fund, to Navneet Kumar is gratefully acknowledged.

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