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Bioactivity of Plectranthus amboinicus: Antioxidant and Antimicrobial Activity

Aman Shajee*
Student, Department of Microbiology, Global Indian International School, Singapore

***Abstract*: This paper presents advances in the bioactivity of borage leaves.**

***Keywords*: Bioactivity, borage leaves.**

1. Introduction

Plants provide a rich source of phytochemicals and phytopharmaceuticals that have been used since antiquity to treat, manage or possibly cure different diseases. Phytochemicals have been studied for their essential role in human disease and health promotion. Many of the bioactive components of plants are phenolic in nature and serve as a defence mechanism to protect plants from photosynthetic stress and add as filter for UV light A myriad of compounds, together with their precursors and reactions products, provide a cocktail, or a soup, which collectively participates in the survival of plants. In 1596, the first Compendium of Materia Medica" was published in China and included some 4773 materials from plants.

Although synthetic drugs have dominated modern western medicine for the past several decades, the leads for synthetic drugs have usually been derived from a plant origin and some 20% of the drug supply is from plant sources. in recent years due to increasing demands of consumers for alternative and preventive health management, there has been a renewed interest in plant material. Thus, the market for supplements and nutraceuticals has expanded exponentially, and the public interest in phytochemicals has been considerable.

Overwhelming epidemiological evidence indicates that a plant-based diet affords protection against the development of certain chronic diseases, particularly cancer. Apart from the macro and micronutrients necessary for normal metabolism, a plant-based diet contains numerous non-nutritive components known as "Phytochemicals", which may also play an important role in health enhancement. As many of the phytochemicals are not well characterized and their mode(s) of action not well established, research on these physiologically active components is currently an area of intense effort.

The disease-preventing potential of phytochemicals in the diet is the main area of scientific interest. Antioxidants have attracted great attention for disease preventive effects because of lipid peroxidation, which can lead to destabilization and disintegration of cell membranes, to many age-related diseases, to aging, and to cancer. Recently, the involvement of free radicals and other oxidants in aging and in several diseases has been investigated in detail. The biochemical literature is full of claims that reactive oxygen species are involved in different diseases.

2. Free Radicals and Reactive Oxygen Species

Free radicals can be defined as species with an unpaired electron The reactivity of free radicals varies from relatively low, as in the case of the oxygen molecule itself, to very high as in the case of the short-lived and highly reactive hydroxyl radical (OH). Fatty acids present in biological membranes as well as in foods are susceptible to attack by highly reactive oxygen species (ROS) such as OH radical, and any reaction or process which forms ROS would definitely stimulate lipid oxidation.

Lipid oxidation is one of the major causes of quality deterioration in lipid-containing foods. It affects the colour, flavour, texture and nutritive value of foods. Oxygen derived free radicals such as superoxide radical (O_2) , hydroxyl radicals (OH) , hydroperoxyl radical (HO_2) and nitric oxide radical (NO) can abstract hydrogen atoms from fatty acid chain of lipid molecules. The occurrence of ROS in foods is inevitable due to the biological nature of foods. ROS also initiate lipid peroxidation in animal and vegetable fats and oils. ROS play an important role in tissue damage in humans. Reaction of ROS with biomolecules such as membrane lipids, proteins and deoxyribonucleic acid (DNA) can provoke irreversible changes in their structure. Membrane lipid peroxidation has been known particularly to associate with many tissue injuries and disease conditions. ROS have been implicated in the development and progression of cancer as well as inflammation and aging.

3. Antioxidants

Incorporation of synthetic antioxidants such as Butylated hydroxyanisole (BHA), Butylated hydroxytouene (BHT), tertiary-butyl hydroquinone (TBHQ), and Propyl gallate (PG) into foods can retard lipid oxidation. However, use of synthetic antioxidants in food products is under strict regulation due to the health hazards caused by such compounds. Due to the possible toxicity of synthetic antioxidants, together with consumers' preference for "natural" products, much natural

*Corresponding author: amanshajee1234@gmail.com

antioxidant research has been undertaken during the past years. As a result, a large number of natural antioxidants have been scrutinized. A multitude of natural antioxidants have already been isolated from different kinds of plant materials such as oil seeds, cereal crops, vegetables, fruits, leaves, roots, spices and herbs. Among these plant materials there is an increasing interest in herbs and spices as sources of natural antioxidants.

Currently materials, which inhibit lipid oxidation, can be obtained from plant materials, food waste, microorganisms and animal cells. The antioxidant activity of plant origin is dependent on the type and polarity of the extracting solvent as well as on the test system and the substrate to be protected by the antioxidant. Natural antioxidative substances usually have a phenolic moiety in their molecular structure They have been found among flavonoids, tocopherols, and catechins. Organic acids, carotenoids, protein hydrolysates and tannins can act as antioxidants or have a synergistic effect when used together with phenolic antioxidants. Flavonoids and other phenolic compounds of plant origin have been reported as scavenger of ROS, thus they are viewed as promising therapeutic drugs for free radical pathologies.

Among natural antioxidants, phenolic antioxidants are in the forefront as they are widely distributed in the plant kingdom. Phenolic compounds occur in oilseeds as the hydroxylated derivatives of benzoic and cinnamic acids, coumarin, flavonoid compounds and lignins. Phenolic antioxidants are reported to quench oxygen derived free radicals as well as the substrate-derived free radicals by donating a hydrogen atom or an electron to the free radical. Several authors have reported that the extracts of various oilseed meals possess antioxidant properties than those observed for synthetic antioxidants at the same concentrations.

Plants produce phenolic compounds to deal with reactive oxygen species (ROS) and substrate derived free radicals produced during the process of photosynthesis. Plant phenolics can delay the onset of lipid oxidation and decomposition of hydroperoxides in food products as well as in living tissues.

The biological effects of phytopolyphenols include antioxidant, antimicrobial, anti-inflammatory and vasodilatory actions. The antioxidant effect of plant phenolics has been studied in relation to the prevention of coronary diseases and cancer, as well as age-related degenerative brain disorders.

4. Antimicrobials

Microbial activity is a primary mode of deterioration of many foods and is often responsible for the loss of quality and safety. Concern over pathogenic and spoilage microorganisms in foods is increasing due to the increase in outbreaks of food borne disease. Currently there is a growing interest to use natural antibacterial compounds like plant extracts of spices and herbs for the preservation of foods as they possess a characteristic flavour and sometimes show antioxidant activity as well as antimicrobial activity.

Resistance to antimicrobial agents such as antibiotics is emerging in a wide variety of microorganisms and multiple drug resistant microorganisms pose serious threat to the treatment of infectious diseases. Hence plant derived antimicrobials have received considerable attention in recent years, several plants are indicated in folk and other traditional systems of medicine as aseptic agents. Though numerous reports have appeared on the microbicidal activity of plants and their secondary metabolites, scientific evaluation of the antimicrobial activity of widely distributed plants still remains an area of intensive investigation.

5. Plectranthus amboinicus (Country Borage)

Indian borage or country borage (Coleus amboinicus, syn, Plectanthrus amboinicus) belongs to the family Lamiaceae. This plant is commonly grown in kitchen garden. The plant is a succulent shrub with large oval fleshy leaves. The leaves are consumed locally in raw form or in preparations such as salads and chutneys. The extracts of the leaves are reported to be employed in certain home remedies / ayurvedic preparations to treat skin disorders, common cold and also diuretics. Literature on the bioactivity of this plant material is not available to the best of our knowledge.

6. Aim of the Investigation

The purpose of this study was to evaluate the bioactivity of different extracts of the leaves of the plant, Coleus amboinicus (Indian borage or Country borage) of the family Lamiaceae, collected locally. The leaves of this plant were examined as potential sources of antioxidant and antimicrobial compounds. Commonly used assays (DPPH radical scavenging, β-carotene bleaching assays) and chromatographic procedures (TLC, HPLC) were employed to investigate the antioxidant activity. The in vitro antibacterial activity of the leaf extracts against selected Gram- positive and Gram-negative as well as certain food borne pathogens and food spoilage bacterial strains were also studied.

7. Materials and Methods

A. Materials

1) Chemicals

β-carotene, tween -20, linoleic acid, gallic acid, vanillic acid, cinnamic acid and DPPH were obtained from Sigma Chemicals Co (St Louis, MO, USA). Anhydrous Zinc chloride and Ferric chloride were obtained from Potomac Chemicals, Singapore. Folin's Ciocalteu phenol reagent and Potassium ferricyanide were obtained from Sisco Research Laboratories (SRL), Singapore. Silica gel was obtained from E-Merck, Singapore. Media employed for antimicrobial study, BHI agar and Nutrient agar were purchased from Toh Chemicals, Singapore. All solvents and other chemicals used were of analytical grade.

2) Plant material

Fresh borage leaves were obtained from home gardens and were either processed fresh or used after drying in an oven at 50°C for 6h. After drying, the leaves were powdered in a blender to obtain dry powder.

3) Bacterial stains

The bacterial stains employed in the studies are listed in the table below. They were obtained from the culture consortium maintained at CFTRI.

Table 1

Bacteria	Type	Properties
Listeria monocytogenes	Gram +ve	Food borne pathogen
Bacillus cereus	Gram +ve	Food borne pathogen
Staphylococcus aureus	Gram +ve	Food borne pathogen
Pseudomonas aeruginosa	Gram -ve	Food spoilage organism
E. coli	Gram -ve	Food borne pathogen

B. Methods

1) Polyphenol standard with Gallic acid

1 mg of gallic acid was weighed and was dissolved in 1 ml of ethanol. From the prepared gallic acid stock solution, 10, 15, 20, 25, 30,35,40,45, and 50 μl were pipetted out into different test tubes. Distilled water was added to make up the volume to 3 ml. Then 0.5 ml of 95% ethanol was added which was followed by the addition of 250 μl of 50% Folin's reagent. It was then vortexed well and incubated at room temperature for 5 minutes. After vortexing 0.5 ml of 5% Sodium carbonate solution was added and again vortex well and then incubated at room temperature for 1 hr. After incubation absorbance was read at 725 nm.

2) Polyphenol estimation

50 μl of the different extracts (10 times diluted), was taken and made up the volume 3 ml with distilled water. To this, 0.5 ml of 95% ethanol was added and shaken well, then 250 ml of 50 % Folin's reagent was added, vortexed and incubated at room temperature for 1 hr. At the end of incubation, the absorbance was read at 725 nm

Total polyphenol concentration in the sample was then calculated as follows:

$$\text{Total polyphenol concentration} = \frac{\text{concentration of standard}}{\text{absorbance of standard}} \times \text{absorbance of sample}$$

3) Chlorophyll estimation in methanolic extract

1 ml of methanolic extract from fresh leaves was taken in a separating funnel 4 ml of distilled water was added to it. 10 ml of diethyl ether was added through the sides of the separating funnel. The separating funnel was inverted thrice, and kept until two layers were formed. The two layers were drained out in two different conical flasks. The bottom layer was poured back into the separating funnel and again 10 ml of diethyl ether was added and the same process repeated till the green colour was completely removed (5 times). The diethyl ether was poured back into the separating funnel and washed with water. Anhydrous sodium sulphate was added to it to absorb traces of water The absorbance of the solution was read at 660 and 642nm.

Total chlorophyll content in the extract was calculated by the formula

- Total chlorophyll mg/I = (7.2x OD at 660 nm)+(16.8x OD at 642.5 nm)
- Chlorophyll a, mg/I = (9.93x OD at 660 nm) - (0.777 × OD at 642.5 nm)
- Chlorophyll b, mg/I = (17.6x OD at 642.5 nm) - (2.81x OD at 660 nm)

4) Micro TLC

Micro TLC was done to standardize the solvent system for separating components in TLC plate.

Approximately 10 gm of silica gel was weighed and to it chloroform was added and mixed well by shaking, until it formed a viscous liquid. Clean microscopic glass slides 2 at a time were held together and dipped into the above solution in such a way that only one of the surfaces of slides got a uniform coat. Coated slides were then kept in hot air oven at 100°C for activation for about 45 minutes.

Total extract of borage leaves was then spotted on the slide with a capillary tube (5 times). It was air dried and was kept in a solvent system containing Hexane: Acetone (10%, 20%, 30%, Hexane: 100% Acetone) in a coplin jar till the solvent front reached the top. Plates after running were developed in an lodine chamber to visualize the spots. 10% Hexane in acetone was found to be the best solvent system.

8. Antioxidant Activity

A. DPPH Assay

50 μl of 10 times and 100 times diluted sample was pipetted out into clean test tubes to which was added 200 ul of 95% ethanol. Then 1.5 ml of dye reagent was added and mixed well by vortexing. The tubes were then kept at room temperature for 30 minutes. After incubation the tubes were centrifuged for 3 minutes at 3000 rpm. Supernatant was then taken and absorbance was read at 517 m against a reagent blank.

Dye reagent: 2,2 Diphenyl 1, Picryl hydrazyl (DPPH) dye was prepared by dissolving 10 mg of dye in 250 ml of 95% ethanol.

B. β-carotene Bleaching Assay

1) Preparation of reagents

Solution I: 3mg of β-carotene was weighed and then dissolved in 20 ml chloroform in a stoppered tube, covered with aluminium foil. This served as the stock solution. It was stored at 4°C.

Solution II: 40 mg Linoleic acid and 400 mg Tween-20 was added to a pre-weighed conical flask which was covered with black paper and to it 3 ml of the β-carotene stock solution was added. The emulsion was mixed well and evaporated under Nitrogen. 100 ml of oxygenated water was added to this evaporated content at the time of the assay

Solution III: Same amount of linoleic acid and Tween-20 as mentioned in solution I without β-carotene was taken in another flask and evaporated 100 ml of oxygenated water was added to this and this served as the blank.

2) Assay procedure

200 μl of extract (TE, AF, AD, MF, MD, RB*) was added to 3 ml of solution II. It was then incubated at 50°C in a water bath for different time periods (0, 10, 20, 40 and 60 minutes). At the end of incubation period, the absorbance was read at 470 nm

Note: For each extract there must be 5 test tubes for taking readings at different time points. TE: Total extract, AF: Acetone fresh, AD: Acetone dry, MF: Methanol fresh, MD: Methanol dry, RB: Reagent blank.

9. Thin Layer Chromatography (TLC)

40 g of silica gel was weighed and dissolved in 100 ml of distilled water. It was mixed well and coated on cleaned TLC glass plates and then kept for air drying After drying, the plates were placed in a hot air oven at 100°C for 45 minutes for activation. After activation, the five extracts (TF, AF, AD, MF, MD) from borage and five standard phenolic acids (vanillic, gallic, ferulic, cinnamic, and protocatechuic) were spotted on the plates using a capillary tube. After spotting, the plates were dried and kept in a chamber, which was saturated with Methanol: ethylacetate (45:105ml) solvent Plates were placed in the chamber until the solvent front reached the top. The plates were then dried and placed in an iodine chamber or sprayed with Ferric Chloride-Potassium ferricyanide solution or β-carotene linoleate solution for visualizing the spots.

A. Preparation of Ferric Chloride-Potassium Ferricyanide Solution

Equal volumes of 1% (w/v) aqueous solution of both salts were prepared and mixed properly to produce an orange-brown-colored reagent. Plates were then sprayed with the above spray. Phenolics, if present, will produce a blue color.

B. β-carotene Linoleate Spray

9 mg of β-carotene was dissolved in 30 ml of chloroform. 2 drops of linoleic acid were added, followed by 60 ml of ethanol.

After running in an ethyl acetate/methanol solvent system, plates were sprayed with the above spray. Plates were kept under fluorescent light for 3 hrs until the orange background colour disappeared. After spraying, bands with persistent orange colour were considered to have antioxidant activity. Colour intensity depends on the antioxidant strength.

Spots, which showed compounds from Methanol fresh and Methanol dry with high antioxidant activity, were scraped and extracted in 2 ml of HPLC grade methanol.

Slurry was then centrifuged at 5000 × g for 3 minutes. 1 ml of the supernatant was evaporated to dryness under a stream of Nitrogen. After evaporation, 100 μl of HPLC grade methanol was added, vortexed and then stored at 4°C and subjected to HPLC analysis.

10. High Performance Liquid Chromatography (HPLC)

A. Sample Preparation

200 μl of MF and MD in 70% methanol was filtered through 0.4μm filter to remove all suspended particles. After filtration it was used for HPLC.

Polyphenols present in the samples were detected by the method of Donata Bandoniene et al., (2002). The chromatographic system consisted of the Shimadzu LC-6A model (Shimadzu, Tokyo. Japan) with a C-18, 5μm silica column (250×4.6mm I.D) at 40°C. The injection system used was a 20μl sample loop. 20μl of scraped and 10μl of original extracts (MF, MD) respectively, were loaded. Detection was done by a UV visible spectrophotometer SPD-6AV set at a wavelength of 280 nm. The separation of antioxidant components was carried out by gradient HPLC with mobile phase composition ranging from 2-80% Acetonitrile with 2% acetic acid in water.

Gradient elution was at a rate of 0.5ml/min with gradient programme (0-40 min, 2-40%, B; 40-50 min 40% B; 50-60 min, 40-50% B) with 2% Acetic acid in water as solvent A and Acetonitrile as Solvent B. The column was equilibrated between injections for 10 min with the initial mobile phase.

11. Antimicrobial Activity

A. OD Method

From the 10^{-4} dilutions, 100 μl cultures of S.aureus and B.cereus were added to test tubes containing 9 ml of sterile nutrient broth. Extracts viz., MD, MF and methanol of varying volumes from 50 μl to 300 μl were added to the tubes. For the different volumes of extracts, control of the same volume, which contains solvent alone, was also maintained. Blank was nutrient broth without culture and extract. After the addition of culture and extracts, test tubes were shaken well and kept for incubation at 37°C for 18 hrs.

After incubation, OD was taken at 560 nm against blank.

Percent inhibition was calculated by the OD reading using the formula:

$$\% \text{ of inhibition} = \frac{\text{Control} - \text{Treated}}{\text{Control}} \times 100$$

B. Pour Plate

To 9 ml of sterile nutrient broth, one loop full of B cereus from a slant was Inoculated under aseptic conditions. Similarly, S.aureus was also sub-cultured. The plates were incubated at 37°C for 18 hrs.

C. Serial Dilution

Nutrient broth inoculated with B.cereus and S aureus, after 18 hrs incubation, was centrifuged at 3,000 pm for 5 min. After centrifugation, the supernatant was removed and the pellets were collected. Pellets were transferred to 9.9 ml sterile saline in another test tube to obtain 10^{-2} dilutions. From 10^{-2} dilutions, 100 μl was transferred to 9.9 ml sterile saline in another test tube to get 10^{-3} dilutions. From 10^{-3} dilution, 1 ml was transferred to 9.9 ml sterile saline to get 10^{-4} dilutions.

From the 10^{-4} dilution of B.cereus and S.aureus, 100 l was transferred to 20 ml sterile nutrient agar. Varying volumes (i.e. 50 ml to 300 ml) of extracts (MD and MF) were added and shaken well and poured into sterile petriplates. For each concentration of extract, separate controls, i.e., methanol of the same volume, were also maintained. After solidification, the plates were kept for incubation at 37°C for 18 hrs.

After incubation, the percentage inhibition was calculated by the colony count method using the following formula:

$$\% \text{ of inhibition} = \frac{\text{Control} - \text{Treated}}{\text{Control}} \times 100$$

D. Agar Well Diffusion

Bacterial cultures, namely Staphylococcus aureus, Pseudomonas aeroginosa, E coli and Bacillus cereus, were grown in slants in nutrient agar media (Himedia, Mumbai,

India; Peptic digest of animal tissue - 5g; NaCI - 5g; beef extract - 1.5g; agar - 20g; final pH 7.2 ± 0.2 plus agar at 15g/I) while Listeria monocytogenes was grown in Brain Heart Infusion (Himedia, Mumbai, India; Calf brain infusion form - 200g; brain heart infusion form - 250g; protease peptone - 10g; dextrose - 2g; NaCI - 5g; Di sodium phosphate - 2.5g; final pH 7.2 + 0.2 plus agar at 15g/L). The cultures were incubated at 37° C. Each bacterial strain was transferred from stored slants at 4-5°C to 1ml nutrient broth and cultivated at 37°C for 24hrs.

100ml of each of the above cultures was transferred separately to 30ml of sterile nutrient agar in different conical flasks under aseptic conditions. It was then shaken well and transferred to sterile petriplates, and kept for solidification. After solidification, wells were made on the nutrient agar. Then 60 μl of different extracts, namely MD, MF, AD, AF and TE and their respective controls, were added into the wells and then pre-incubated at 4°C for 3 hrs to allow the test material to diffuse into the agar and later incubated at 37°C for 18hrs. The Individual plates were examined for the zone of clearance around the individual wells. The diameter of the zone of clearance, if any, thus formed was measured, and the arbitrary units/mL (AU/ml) was calculated as follows:

$$\text{AU/ml} = \frac{\text{Diameter of the zone of clearance}}{\text{Volume taken in the well}} \times 1000$$

12. Results and Discussions

A. Chlorophyll Content in Methanolic Extract of Fresh Borage Leaves

Chlorophyll content was estimated in the methanolic extract of fresh leaves and it was found that the extract contained 0.19mg/ml chlorophyll a and 0.009205 mg/ml chlorophyll b.

B. Phenolic Compounds in Borage Leaf Extracts

The total phenolic content (TPC) of borage leaf extracts is presented in Table 2. The total phenolic contents were measured as gallic acid equivalent / g of fresh or dried leaves from which the extract was prepared. The gallic acid standard showed linearity and this graph (Table 2, Fig. 1) was employed for calculating the TPC.

Table 2
Polyphenol standard curve with gallic acid

Gallic acid Volume taken (μl)*	OD at 725 nm
10	0.143
15	0.181
20	0.291
25	0.386
30	0.440
35	0.522
40	0.532
45	0.583
50	0.765

Among the five different extracts, the crude leaf extract contained 0.31 mg GA/g, while the methanolic extract of both fresh and dried leaves showed the highest TPC (7.5-9.6 mg GAE/g). Acetone extracts showed TPC of 0.8-1 mg GAE/g.

TLC analysis of the various extracts revealed multiple compounds in each extract (Fig. 2). However, based on R values none of the standard phenolics nor chlorophyll could be identified in the extracts.

HPLC analysis was carried out for only methanolic extract of fresh (MF) and dried leaves (MD). Both the extracts revealed at least fifty to sixty compounds (Fig. 7). The UV spectrum of the two extracts revealed absorption peaks between 272-322 nm, characteristic of the phenolic acids.

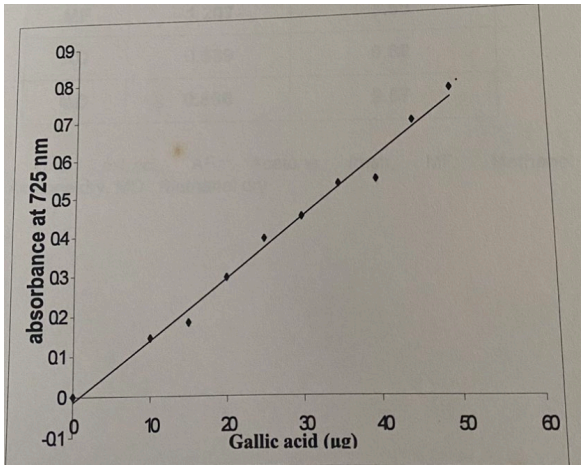

Fig. 1. Standard curve of gallic acid

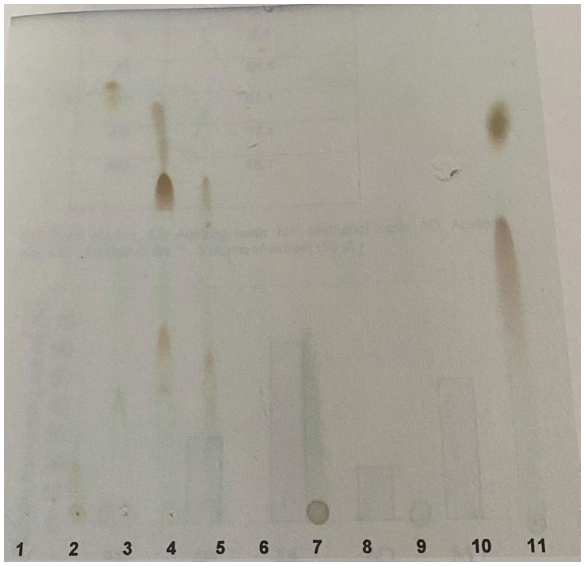

Fig. 2. Thin layer Chromatography of various extracts of borage leaves, standard phenolic acids and chlorophyll

1) TE- Total Extract
2) AF- Acetone Fresh.
3) AD- Acetone Dry
4) MF- Methanol Fresh
5) MD- Methanol Dry
6) Ferulic acid
7) Gallic acid
8) Vanillic acid
9) Protocatechuic acid
10) Cinnamic acid
11) Chlorophyll

C. Antioxidant Activity of Borage Leaf Extracts

1) Free radical scavenging activity of DPPH assay

The model system of scavenging DPPH free radical is yet another simple method to evaluate the antioxidative activity of an antioxidant. It is accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen donating ability. The concentration of antioxidant need to decrease by 50% of the initial substrate concentration (EC_{50}) is a parameter

Table 2
Total Polyphenol content of various extracts of borage leaves

Extract	OD at 725 nm	Total phenol concentration (GAE mg/g fresh or dried leaves)
TE (Total Extract)	0.039	0.31
AF (Acetone Fresh)	0.450	1.00
MF (Methanol Fresh)	1.207	7.50
AD (Acetone dry)	0.339	0.80
MD (Methanol dry)	0.866	9.67

widely used to measure the antioxidant power of any compound. Lower the EC_{50}. higher the antioxidant power. The accessibility of the radical centre of DPPH to each polyphenol could also influence the order of the antioxidant power obtained (Yoshida et al., 1989).

The radical scavenging activities of the different extracts are shown in Table 3, Fig.3. The extent of the free radical (DPPH) scavenging activity ranged between 6 to 61% at equal volume of extracts. Methanolic extracts generally exhibited the highest antiradical activity, followed by the acetone extracts. The methanol extract of fresh leaves exhibited the maximum radical scavenging activity.

A good correlation was evident between the TPC and radical scavenging activity of the extracts (Table 2 and 3). While the order of TPC was: MD> MF> AF> AD> TE, the DPPH radical scavenging activity was: MF>MD>AF>AD>TE. Hence among all the extracts analysed, a significant phenolic content and radical scavenging activity were found for the methanolic extracts of both fresh and dried leaves.

Table 3
DPPH radical scavenging activity of various extracts of borage leaves

Extract*	Radical scavenging activity (%)
TE (Total Extract)	5.9
AF (Acetone Fresh)	28.4
MF (Methanol Fresh)	61.1
AD (Acetone dry)	17.4
MD (Methanol dry)	46.7

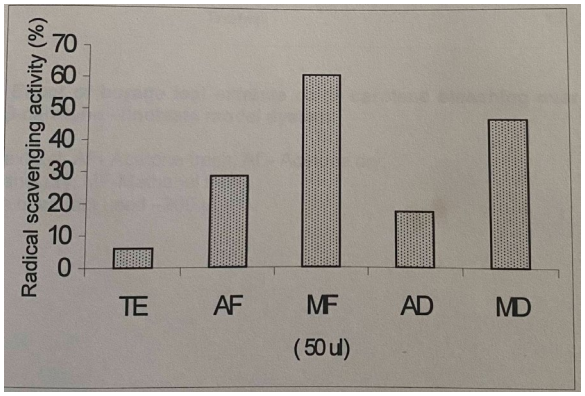

Fig. 3. Radical scavenging activity of the extract of borage leaves in various solvents by DPPH method

D. Antioxidant Activity: β-carotene Bleaching

The antioxidant assay using the discoloration of β-carotene is another widely used assay system because β-carotene is extremely susceptible to free radical mediated oxidation and β-carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. The linoleic acid free radical, formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsaturated β-carotene models. As β-carotene molecules lose their double bonds by oxidation, the compound loses its chromophore and characteristic orange color which can be monitored spectrophotometrically. The presence of any antioxidant can hinder the extent of β-carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in the system. In the present study, all the extracts showed varying degrees of antioxidant activity in β-carotene bleaching assay, when compared with the control. The antioxidant activity determined by the β-carotene bleaching method revealed that all the extracts except the crude extract possessed significant antioxidant activity (Fig. 4). The extent of inhibition of β-carotene bleaching by the four extracts ranged from 8 - 77%.

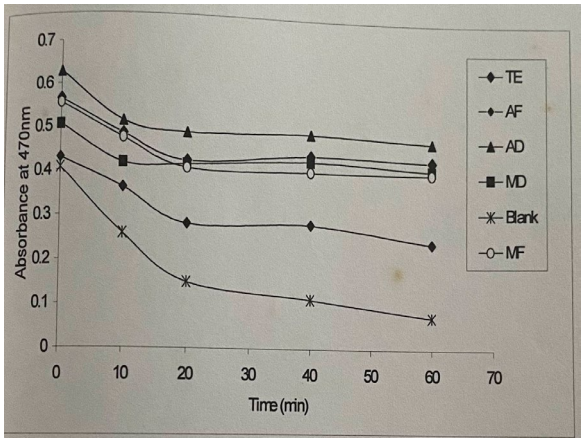

Fig. 4. Effect of borage leaf extracts on β-carotene bleaching over time in a β-carotene-linoleate model system *(Volume of extract used -200 µl)*

E. Antioxidant Activity in situ in TLC with β-carotene-Linoleate Spray

The relative antioxidant activity of band components on the developed TC plates was determined using the β-carotene-linoleate spray. Fig.5 shows the results when a developed plate was sprayed with a β-carotene-linoleate emulsion. Two different spots with intense yellow colour were evident in MF and MD extracts even after 3 hrs exposure to light. This was an indication of the presence of one or more antioxidant compounds in these extracts.

F. HPLC of the Antioxidant Spots

The HPLC of MF and MD showing antioxidant activity (Fig. 5, 1 lanes 3 and 4 and 2 lanes 5 and 6) were isolated and subjected to preparative HPLC. Methanolic extract of fresh leaves showed 4-5 sharp peaks (Fig. 6) while methanolic extract of dried leaves showed 3 sharp peaks (Fig. 7). These results indicate that 3-5 compounds were involved in the antioxidant activity of the methanolic extracts of borage leaves.

Fig. 5. Thin layer Chromatography of various extracts of borage leaves after spraying with β-carotene linoleate solution,

plates were exposed with light for 3hrs.

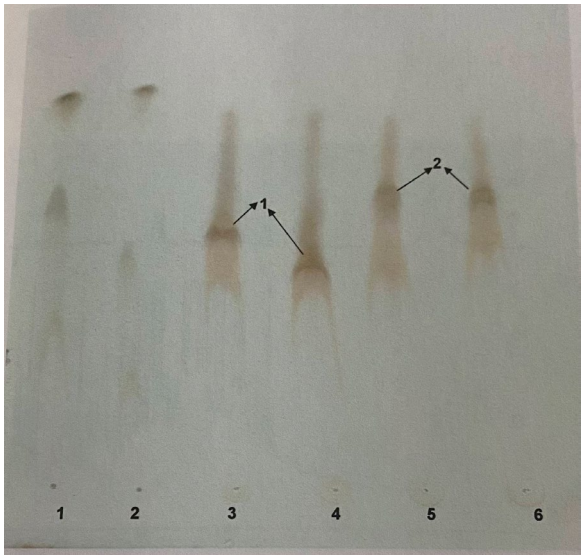

Fig. 5. Thin layer Chromatography of various extracts of borage leaves after spraying with β-carotene linoleate solution, plates were exposed with light for 3hrs (Spots 1 and 2 are scraped for HPLC)

1) AF- Acetone Fresh
2) AF- Acetone Fresh
3) MF- Methanol Fresh
4) MF- Methanol Fresh
5) MD- Methanol Dry
6) MD- Methanol Dry

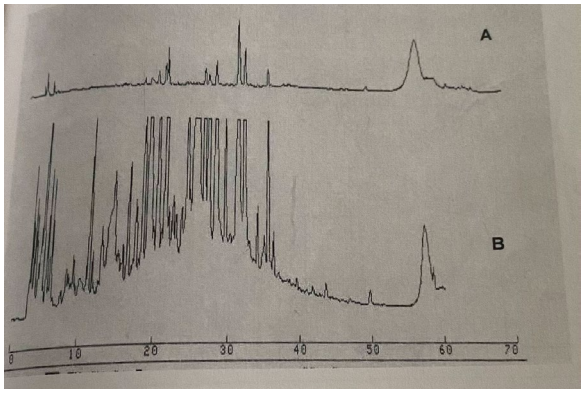

Fig. 6. HPLC profile of methanolic extract of fresh borage leaves
A) Methanolic extract of spots showing antioxidant activity in Fig. 5, lanes 3,4
B) Original methanolic extract of fresh leaves

G. Antibacterial Activity of Extracts of Borage Leaves

1) OD method

Table 4
Antimicrobial activity of methanolic extract of fresh borage leaves against S. aureus using OD method

Volume of extract (Methanol Fresh)	% inhibition
50μl	16.4
100μl	37.6
150μl	46.3
200μl	51.3
250μl	89.6
300μl	98.9

(Extract of fresh leaves with methanol 50-300 μl: 1g-6g of fresh leaf)

The results of this bioassay showed that methanolic extract of fresh borage leaves possessed strong antimicrobial activity against S. aureus (Table 4, Fig. 8) and B. cereus (Table 5, Fig. 9). The extract exhibited a concentration - response in both the bacterial strains. However, the extract seemed to be more effective against S. aureus with nearly 100% inhibition of growth at 300 μl of the extract (equivalent to 6 g of fresh leaves).

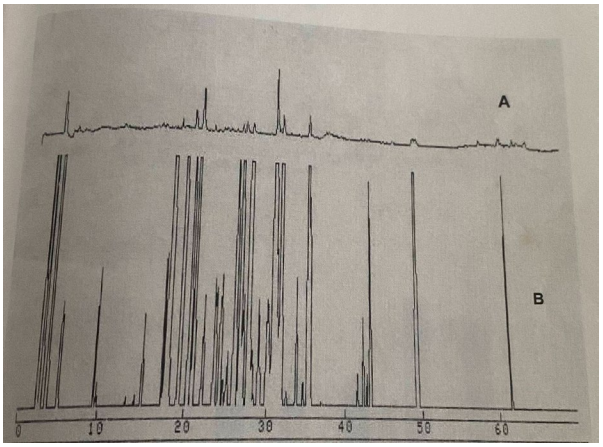

Fig. 7. HPLC profile of methanolic extract of fresh borage leaves
A) Methanolic extract of spots showing antioxidant activity in Fig. 5, lanes 5,6
B) Original methanolic extract of fresh leaves

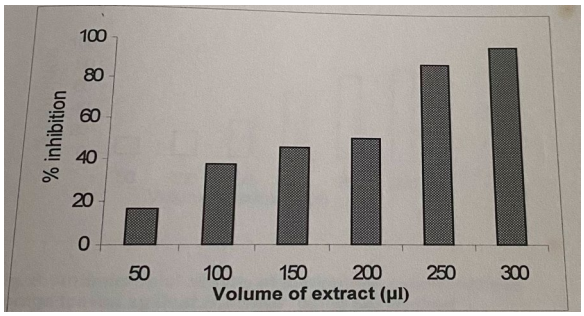

Fig. 8. Antimicrobial activity of methanolic extract of fresh borage leaves against S.aureus using OD method

Table 5
Antimicrobial activity of methanolic extract of fresh borage leaves against B. cereus using OD method

Volume of extract (Methanol Fresh)	% inhibition
50μl	14.8
100μl	21.4
150μl	32.5
200μl	60.8
250μl	79.4
300μl	86.4

(Extract of fresh leaves with methanol 50-300 μl: 1g-6g of fresh leaf)

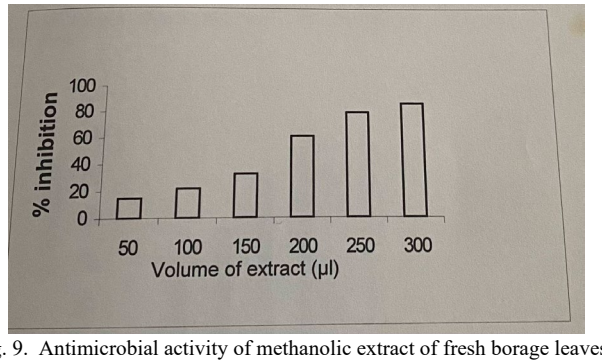

Fig. 9. Antimicrobial activity of methanolic extract of fresh borage leaves against B.cereus using OD method

2) Pour plate method

Comparative antimicrobial activity of methanolic extract of fresh leaves against S. aureus and B. cereus by pour plate method revealed again that MF was more effective against S. aureus. In this bioassay also, a concentration -response was evident in both the bacterial strains (Table 6, Fig. 10). While 100% inhibition in S. aureus growth was seen with 200 μl of MF, 350 μl of MF was required to result in 100 % growth inhibition of B. cereus.

Being more sensitive to borage extract, S. aureus was tested for its response against methanolic extract of fresh and dried leaves. It was evident from the pour plate method that MF was more effective against S. aureus than MD (Table 7, Fig. 11).

Table 6
Antimicrobial activity of methanolic extract of fresh borage leaves against S.aureus and B.cereus using pour plate method

Volume of extract (Methanol Fresh)	%inhibition	
	S.aureus	B.cereus
50μl	30.4	9.4
100μl	54.2	29.0
150μl	78.8	46.8
200μl	100.0	65.8
250μl	Not Done	78.0
300μl	Not Done	91.4
350μl	Not Done	100.0

(Extract of fresh leaves with methanol 50-350 μl: 1g-7g of fresh leaf)

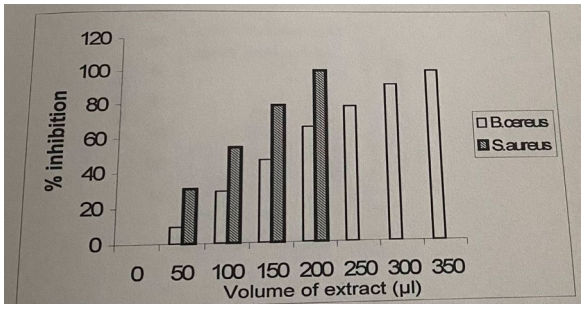

Fig. 10. Antimicrobial activity of methanolic extract of fresh borage leaves against S.aureus and B.cereus using pour plate method

Table 7
Comparative antimicrobial activity of methanolic extract of fresh and dried borage leaves against S.aureus and B.cereus using pour plate method

Volume of extract		% inhibition
50μl	Methanol Fresh	30.4
	Methanol Dry	10.9
100μl	Methanol Fresh	54.2
	Methanol Dry	37.5
150μl	Methanol Fresh	78.8
	Methanol Dry	46.2
200μl	Methanol Fresh	100.0
	Methanol Dry	87.0

(Extract of fresh leaves with methanol 50-200 μl: 1g-4g of fresh leaf)
(Extract of dry leaves with methanol 50-200 μl: 60mg-240mg of dry leaf powder)

3) Agar well diffusion method

The antibacterial activity of acetone extract of fresh and dried borage leaves was tested against the various bacterial strains by agar well diffusion method, and the diameter of zone of growth inhibition was measured. This was also compared with the standard antibiotics viz., Methicillin and Ampicillin against B. cereus, by the disc diffusion method.

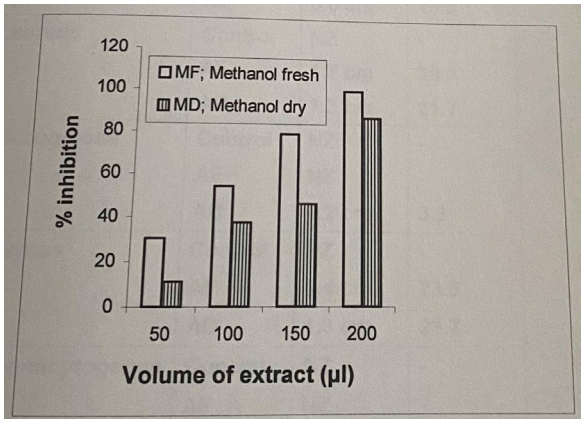

Fig. 11. Comparative antimicrobial activity of methanolic extract of fresh and dried borage leaves against S.aureus using pour plate method

In this bioassay, it was found that the acetone extracts was significantly effective against S. aureus and B. cereus as determined by the diameter of zone of inhibition (Table 9, Fig. 12A and B). The values were comparable to that affected by the standard antibiotics (Table 8, Fig. 14A).

While there was no difference in the effect among AF and AD, the sensitivity of the bacterial strains to the acetone extracts was:

S. aureus > B. cereus > E. coli > P. aeruginosa > L. monocytogenes.

A similar trend was also evident against the bacterial strains when tested with methanolic extract of fresh and dried leaves (Table 10, Fig. 13 A, B, C; Fig. 14 A, B, C).

Overall, methanolic extracts were more effective than acetone extracts against the bacterial strains in this bioassay.

Table 8
Antimicrobial activity of selected antibiotics against B.cereus using disc diffusion method

Organism	Antibiotic	Culture concentration (μl)	Diameter of zone(cm)
B.cereus	Methicillin	100	2.4
B.cereus	Ampicillin	100	1.3

Table 9
Antimicrobial activity of acetone extract of fresh and dried borage leaves using disc diffusion method

Organism**	Extract*	Diameter of Zone(cm)	AU/ml
E.coli	Control	NZ	-
	AF	NZ	-
	AD	0.8	13.3
S.aureus	Control	NZ	-
	AF	1.7	28.3
	AD	1.3	21.7
P.aeruginosa	Control	NZ	-
	AF	NZ	-
	AD	0.2	3.3
B.cereus	Control	NZ	-
	AF	1.4	23.3
	AD	1.3	21.7
L.monocytogenes	Control	NZ	-
	AF	NZ	-
	AD	0.1	1.6

[Volume of culture employed -100 μl; + Volume of extract employed – 60 μl
NZ: No zone of inhibition, Control: Acetone; AF: Acetone Fresh; Extract of fresh leaves with Acetone (60 μl: 0.36 g of fresh leaf); AD: Acetone Dry; Extract of dry leaves with Acetone (60 μl :0.036 g of dry leaf powder)]

Table 10
Antimicrobial activity of methanolic extract of fresh and dried borage leaves using agar well diffusion method

Organism**	Extract*	Diameter of Zone(cm)	AU/ml
E.coli	Control	NZ	-
	MF	1.1	18.3
	MD	0.5	3.3
S.aureus	Control	NZ	-
	MF	1.8	30.0
	MD	1.6	26.7
P.aeruginosa	Control	NZ	-
	MF	0.9	15.0
	MD	0.5	3.33
B.cereus	Control	NZ	-
	MF	1.9	31.6
	MD	1.7	28.3
L.monocytogenes	Control	NZ	-
	MF	1.5	25.0
	MD	1.1	18.3

[Volume of culture employed -100 μl; + Volume of extract employed - 60μl
NZ: No zone of inhibition, Control: Methanol; MF: methanol Fresh; Extract of fresh leaves with methanol (60 μl: 0.36 g of fresh leaf); MD: Methanol Dry; Extract of dry leaves with methanol (60 μl :0.036 g of dry leaf powder)]

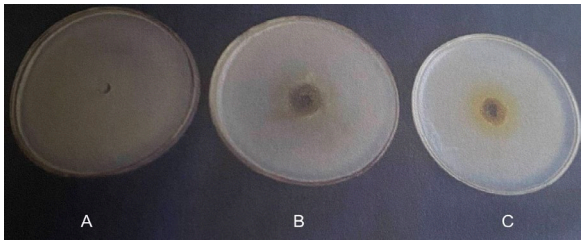

Fig. 12. Antibacterial effect of borage leaf extracts against B. cereus (Growth inhibition by, (a) Acetone; (b) Acetone extract from dry leaves; (c) Acetone extract from fresh leaves)

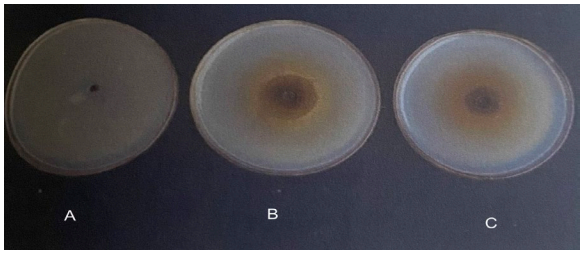

Fig. 12(a). Antibacterial effect of borage leaf extracts against B. cereus (Growth inhibition by, (a) Methanol; (b) Methanol extract from dry leaves; (c) Methanol extract from fresh leaves)

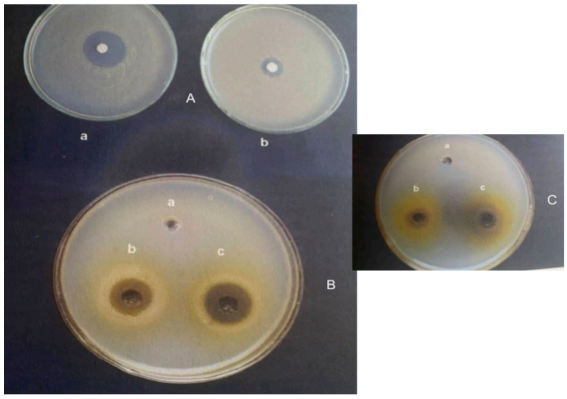

Fig. 13. Antibacterial effect of –
A: Certain antibiotics against B. cereus by disc diffusion method (a) Methicillin (b) Ampicillin, B: Methanol extract of borage leaves against S. aureus, (a) Methanol (b) Methanol Dry (c) Methanol fresh, C: Methanol extract of borage leaves against E. coli (a) Methanol (b) Methanol Dry (c) Methanol fresh

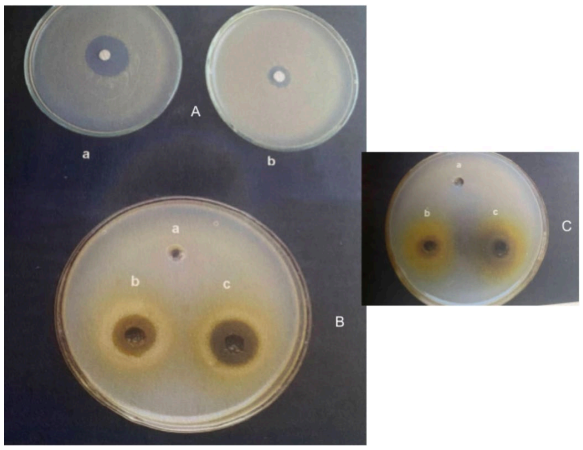

Fig. 14. Antioxidant effect of methanolic extracts of borage leaves against–
A: Bacillus cereus (a) Methanol (b) Methanol extract from fresh leaves (c) Methanol extract from dried leaves, B: Listeria monocyogenes (a) Methanol (b) Methanol extract from fresh leaves (c) Methanol extract from dried leaves, C: Pseudomonas aeruginosa (a) Methanol (b) Methanol extract from fresh leaves (c) Methanol extract from dried leaves

13. Conclusion

1. The extracts of Indian borage leaves - crude, acetone and methanolic contained significant amounts of total polyphenols. Methanolic extract of fresh and dried leaves contained maximum polyphenols.
2. The methanolic extract showed absorption peaks between 272-322 nm and, on TLC, showed multiple spots characteristic of phenolics, which were unidentifiable. HPLC revealed 50-60 peaks in these extracts.
3. The methanolic extracts exhibited significant radical quenching and antioxidant activity. Overall, the radical quenching activities of the extracts correlated with the total phenolic content.
4. The antioxidant activity of the methanolic extract could be attributed to a few compounds, as evidenced by the HPLC of the antioxidant fractions.
5. Methanolic extract of fresh borage leaves also possessed strong antimicrobial activity against S.aureus and B.cereus. However, S aureus was more sensitive to the extract.
6. Similarly, acetone extracts of borage leaves were also antimicrobial against S.aureus and B.cereus.
7. Methanolic extracts were more effective than acetone extract and were comparable in effect to that of methicillin and ampicillin.
8. These results clearly indicate that Indian borage leaves contain bioactive compounds possessing the significant potential to retard lipid peroxidation in biological food systems. They also possess the ability to prevent food spoilage and combat certain bacterial infections.

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