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# *In vitro* antibacterial activity as related to antioxidant property of some ethnomedicinal plants

**Jayati Saha and Prabir Kumar Sarkar***
*Microbiology Laboratory, Department of Botany, University of North Bengal, Siliguri-734 013*

**Abstract**

The present study attempted to evaluate *in vitro* antibacterial and antioxidant activities of extracts from some ethnomedicinal herbs and to correlate among the parameters. The antibacterial activity was assayed using agar-disc diffusion method against seven bacterial species. Their total flavonoids content (TFC) and ferric reducing power (RP) were also evaluated. Herb extracts with high TFC exhibited a good antibacterial activity against the bacteria at low concentrations. The Gram-positive bacteria were more sensitive to the tested extracts than the Gram-negative ones. While *Staphylococcus aureus* was maximally inhibited, *Escherichia coli* was most resistant. Against each bacterium, antibacterial activity was positively correlated ($r = 0.60–0.87$) with TFC of the tested extracts. Positive correlations were also obtained between antibacterial and antioxidant activities ($r = 0.60–0.96$) as well as between TFC and antioxidant activity ($r = 0.91$) of the extracts. Thus, antibacterial and antioxidant activities of the tested extracts were closely associated with their flavonoid constituents.

**Keywords:** Medicinal herb; Flavonoid; Antibacterial; Antioxidant

## Introduction

Microbial contaminations from various sources are a major concern throughout the world. With the emergence of new antibiotic-resistant of bacteria, the scenario is becoming quite alarming. A growing demand for natural antimicrobial agents and elimination of synthetic preservatives in food has provided an increased impetus to explore different natural sources. In this context a large number of plant sources, including many medicinal herbs, spices, vegetables and foods, have been explored for their antimicrobial and antioxidant activities (Shan *et al.,* 2007). More recently, medicinal plant extracts were developed and proposed for use in food as natural antimicrobials (Hsieh *et al.,* 2001). Phenolics are a catagory of phytonutrients that exert antimicrobial and antioxidant properties (Shan *et al.,* 2007). They can be classified into simple phenols, phenolic acids, hydroxycinnamic acid derivatives and flavonoids. Flavonoids, ubiquitous to plants, are active constituents of preparations used from ancient times in curing several diseases (Havsteen, 1983). A strong structure-function relationship exists among these groups of compounds, *viz.* the position and number of hydroxyl groups in the phenolic compounds are possibly related to their relative toxicity to microorganisms, since increased hydroxylation results in increased toxicity (Cowan, 1999). The systemic screening of antimicrobial and antioxidant plant extracts represents a continuous effort to find new compounds with the potential to act against multidrug-resistant spoilage and pathogenic microorganisms.

The Eastern Himalayan region is one of the hotspots in plant biodiversity with a large number of medicinal herbs (Ahmedulla and Nayar, 1999) and in diverse human race too, with a large number of tribes sharing a common habitat. Eventually several plant species of this region have been tagged as medicinal plants and a variety of curative properties have been attributed to the plants used as folk medicines.

Our work aims in resolving (1) *in vitro* antimicrobial profile of the methanolic extracts of five medicinal plants of Darjeeling Himalaya against a panel of bacteria, along with their flavonoid content, and antioxidant activity (reducing power, RP); (2) relationship between antibacterial activity and TFC to understand whether the flavonoids are responsible for the activity; and (3) correlation between antibacterial activity and antioxidant capacity of the extracts, if any.

**Corresponding Author:*
Email: pksarkar.nbu@gmail.com

## Materials and methods

*Microorganisms*

The bacterial strains used in this study were *Bacillus subtilis* DK-W1, *Escherichia coli* MTCC 118, *Staphylococcus aureus* MTCC 1430, *Listeria monocytogenes* MTCC 839, *Bacillus pumilus* HWC 86, *Bacillus licheniformis* HWC 84 and *Bacillus cereus* HWC 88. All these strains were obtained from the Microbial Culture Collection of the University of North Bengal.

*Sampling*

Fresh plant specimens (Table 1) were collected in air-tight polyethylene sampling bags and brought to the laboratory as soon as possible. They were cleaned, treated with 8 g $HgCl_2$ $l^{-1}$ ethanol, pressed and dried using blotting paper, and finally mounted onto herbarium sheets. The samples were identified, taking the help from Plant Taxonomy and Environmental Biology Laboratory, Department of Botany, University of North Bengal.

*Preparation of extracts*

Samples of collected plant parts were dried in a hot air oven for 24-48 h at 60°C and pulverized using a waring blender (Bajaj, India). Each of the powders (10 g) was soaked in 10 vol. of methanol (SRL 132977) for 24 h at room temperature with intermittent shaking, and the supernatant decanted. The extraction process was repeated three times, using fresh solvent. Individual extracts were pooled and filtered through a Whatman No. 1 paper, evaporated *in vacuo* at 40°C and lyophilized (Eyela FDU-506 freeze dryer). The lyophilized extracts were stored in a vacuum desiccator at 4°C. Prior to use, the lyophilized extracts were dissolved in methanol.

*Disc diffusion assay*

Sterilized Whatman No. 1 filter paper discs (5.5 mm) were soaked with different concentrations of herbal extracts (final concentration, 0.1-8.0 mg lyophilized extract $disc^{-1}$) and air dried in a laminar air flow cabinet. Disc diffusion assay was carried out using seven bacterial strains. A loopful of a 24 hold culture was inoculated into tryptone soya broth (HiMedia M011). After 6-8 h. growth on a rotary shaker (200 rpm), the cell concentration was adjusted to $10^8$ $ml^{-1}$, and used for surface seeding using a sterile swab on Mueller-Hinton agar (HiMedia M173) plates. The seeded plates were left to stand for 15 min, impregnated with filter paper discs containing desired concentrations of the herbal extracts and incubated for 18-24 h at 37°C. The assay was carried out in triplicate. A clear zone of inhibition surrounding the discs with a diameter greater than 5.5 mm was considered to be positive.

*Assay of flavonoids*

The TFC was estimated following the method of Zhinshen et al. (1999). A 400 µl-aliquot of the extract (1 mg $ml^{-1}$) was added with 30 µl aqueous solution of 50 mg $NaNO_2$ (Merck 61754305001046) $ml^{-1}$. After incubation for 5 min at 25°C, 30 µl aqueous solution of 100 mg $AlCl_3.6H_2O$ (Merck 80108202501730) $ml^{-1}$ was added, followed after 6 min by addition of 200 µl

Table 1. Plants investigated for potential antibacterial activity

| Scientific name | Family | Local name | Part used | Ethnomedicinal use |
|---|---|---|---|---|
| *Cynodon dactylon* (L.) Pers. | Poaceae | Dubo | Aerial part | Indigestion, cut and wounds |
| *Enydra fluctuans* DC. | Asteraceae | Hincha | Aerial part | Skin disease, liver problem, diabetes and bronchitis |
| *Gloriosa superba* L. | Colchicaceae | Bikh-phool | Leaf | Rheumatism, skin disease and leprosy |
| *Leucas indica* (L.) W.T.Aiton | Lamiaceae | Dulphe jhar | Leaf | Sinusitis and nasal infections |
| *Ocimum tenuiflorum* L. | Lamiaceae | Babari-phool | Inflorescence | Cough and throat infections |

aqueous solution of 1 mol $l^{-1}$ NaOH (Merck 61757305001046). The mixture was diluted to 1 ml with water, and the absorbance at 510 nm was read. The TFC value was expressed in terms of mg epicatechin equivalents $g^{-1}$ lyophilized extract, using a standard curve of epicatechin (Sigma E1753).

*Assay of reducing power*

The ability of the extracts to reduce Fe(III) was assessed according to the method of Oyaizu (1986). A 1.0-ml aliquot of lyophilized extract solution was mixed with 2.5 ml of 0.2 mol $l^{-1}$ phosphate buffer (pH 6.6) and 2.5 ml aqueous solution of 10 g potassium ferricyanide (HiMedia RM1034) $l^{-1}$. The mixture was incubated for 20 min at 50°C, added with 2.5 ml aqueous solution of 100 g trichloroacetic acid (SRL 204842) $l^{-1}$ and centrifuged at 1200g for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 1.0 g $FeCl_3$ (SRL 64765) $l^{-1}$, and the absorbance was measured at 700 nm. The RP was expressed as mg ascorbic acid equivalents $g^{-1}$ lyophilized extract, using the standard curve of ascorbic acid (SRL 149100).

*Statistical analysis*

The data were analysed using Minitab version 15.0 (Minitab Inc. State College, PA, USA). The results were expressed as mean ± standard error. Values were considered significant at $P < 0.05$. To assess the relationship between the activities and the flavonoids content, Pearson's correlation coefficients were calculated with 95% confidence level.

**Results**

*Flavonoids content*

While the highest TFC was found in *Leucas indica* closely followed by *Ocimum tenuiflorum*, *Cynodon dactylon* had the lowest TFC among the herbs tested (Table 2).

*Antibacterial activity*

In general, Gram positive bacteria were most susceptible to the majority of the extracts tested (Table 2). On the other hand, the Gram negative bacterium (*E. coli*) was quite resistant as it was inhibited by only two of the five extracts, that too at higher concentrations. The antibacterial inhibitory power of *Enydra fluctuans* was also good as it could control about 71% of the bacteria at a concentration of ≤ 1.0 mg $disc^{-1}$.

*Relationship between flavonoids and antibacterial and antioxidant activities*

Extracts of *L. indica* and *O. tenuiflorum* with high TFC showed excellent antibacterial activity, while the extract of *C. dactylon* with poor TFC showed no effect (Table 2). The antimicrobial patterns of all the bacteria used were not significantly correlated with TFC of the herbs. Figure 1 shows regression equations and correlation coefficients ($r$) between antibacterial activity and TFC ($P <$

Table 2. Total flavonoids content (TFC), reducing power (RP) and minimum inhibitory concentration (MIC) of the extracts from medicinal herbs*

| Scientific name | TFC† | RP‡ | MIC (mg lyophilized extract $disc^{-1}$)§ | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | *Ec* | *Lm* | *Bc* | *Bs* | *Bl* | *Bp* | *Sa* |
| *Cynodon dactylon* | 4.2 ±0.3 | 22.7±1.88 | >8.0 | >8.0 | 7.0 | >8.0 | >8.0 | >8.0 | >8.0 |
| *Enydra fluctuans* | 14.1±0.4 | 822.5±5.27 | >8.0 | 1.0 | >8.0 | 1.0 | 0.2 | 0.5 | 0.2 |
| *Gloriosa superba* | 10.2±0.7 | 388.1±3.08 | 3.0 | >8.0 | 7.0 | 6.0 | 2.0 | 7.0 | 1.0 |
| *Leucas indica* | 25.4±1.5 | 927.2±2.28 | >8.0 | 7.0 | 0.5 | 0.1 | 0.2 | 0.2 | 0.2 |
| *Ocimum tenuiflorum* | 25.0±1.8 | 868.1±7.40 | 7.0 | 5.0 | 2.0 | 6.0 | 1.0 | 1.0 | 0.5 |

* Values indicate mean ± SEM of triplicate sets; † mg epicatechin equivalent $g^{-1}$ lyophilized extract; ‡ mg ascorbic acid equivalent $g^{-1}$ lyophilized extract; § *Ec*, *Escherichia coli*; *Lm*, *Listeria monocytogenes*; *Bc*, *Bacillus cereus*; *Bs*, *Bacillus subtilis*; *Bl*, *Bacillus licheniformis*; *Bp*, *Bacillus pumilus*; *Sa*, *Staphylococcus aureus*

0.05). The *r* values were between 0.60 and 0.87. *B. cereus* showed maximum correlation closely followed by *B. pumilus*. *S. aureus* and *B. licheniformis* showed moderate correlation, while *B. subtilis* was least correlated. Figure 2 shows the correlation between antibacterial activity and RP (antioxidant activity) of the extracts ($P < 0.05$). The *r* values were between 0.60 and 0.96. *B. pumilus*, *B. licheniformis* and *S. aureus* had high positive correlations, while *B. subtilis* was moderate and *B. cereus* was the lowest. Figure 3 shows high positive correlation ($P < 0.05$) between flavonoid content and RP.

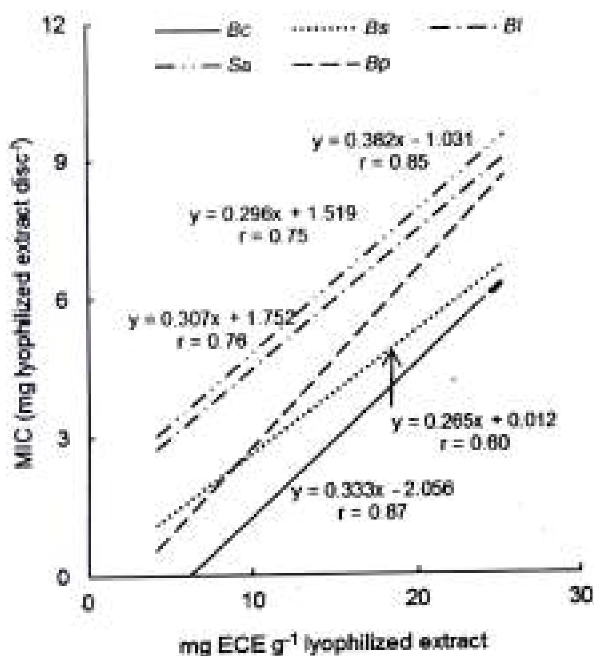


Fig. 1. Regression lines showing relation between total flavonoids content (epicatechin equivalent, ECE) and minimum inhibitory concentration (MIC)

## Discussion

Agar disc diffusion method is extensively used to investigate the antibacterial activity of natural substances and plant extracts. The assays are based on the use of discs as reservoirs containing solutions of substances to be examined (Gülçin *et al.*, 2003). Therefore, this method was selected for antibacterial assay. The results show that the Gram positive bacteria are more susceptible than the Gram negative ones. This finding was in consistence with that in literature (López *et al.*, 2005; Shan *et al.*, 2007). This can be explained as there lies a significant difference in the outer layers of the groups of

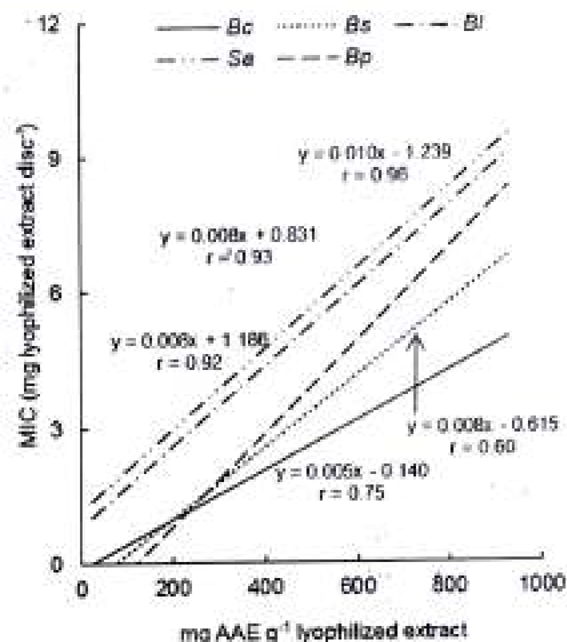


Fig. 2. Regression lines showing relation between antioxidant activity (ascorbic acid equivalent, AAE) and minimum inhibitory concentration (MIC)

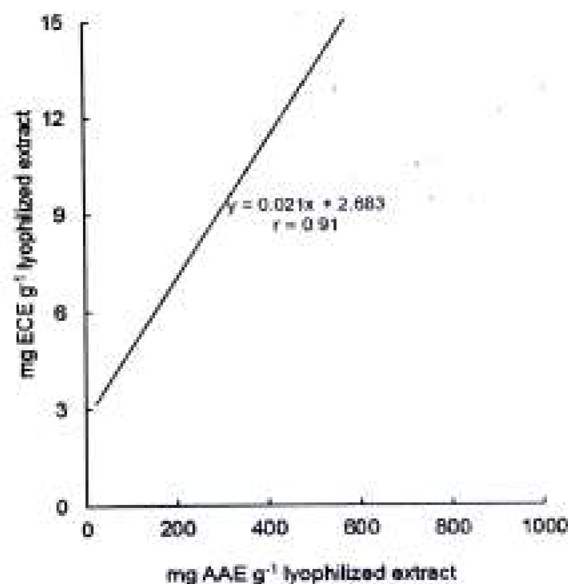


Fig. 3. Regression line showing relation between total flavonoids content (epicatechin equivalent, ECE) and antioxidant activity (ascorbic acid equivalent, AAE) of extracts from five medicinal plants used in the study

bacteria and the Gram negative ones possess an outer membrane and a unique periplasmic space (Duffy and Power, 2001). Antibacterial activity, flavonoids content and antioxidant activities of a number of medicinal herbs have been documented extensively in literature, however, each group had used different assay protocols, different microbial species and varied herb samples. This makes it difficult to establish any relationship between the activities. Shan *et al.* (2007) reported a high positive correlation between the phenolic content and antibacterial and antioxidant activities of medicinal herbs and spices.

The extract of *O. tenuiflorum* inflorescence was the most potent as it inhibited all the bacteria. While both *L. indica* and *G. superba* inhibited 85% bacteria employed in the study, *L. indica* inhibited them at lower concentrations as compared to *G. superba*, which on the other hand controlled *E. coli* at a lower concentration. *Ocimum* spp. are rich in volatile monoterpenes, essential oils, several flavonoid glycosides, flavones, nevadensin, xanthomicrol and gardenin B (Ultee *et al.*, 1999; Grayer *et al.*, 2002). *Leucas* spp. possess several flavonoids such as leucasin, which is reported to have a strong antioxidant potential (Meghashri *et al.*, 2010). Though the flavonoid content of *G. superba* was much lower than that of *L. indica*, its efficient antibacterial activity may be due to the presence of alkaloids, colchicine and colchicoside, which were reported to have antimicrobial properties (Khan *et al.*, 2008).

It is well known that plant polyphenols in general and flavonoids in particular possess antimicrobial and antioxidant activities. Inhibition of nucleic acid synthesis, cytoplasmic membrane function and energy metabolism are the different mechanisms by which flavonoids exert antibacterial actions (Tim Cushnie and Lamb, 2005; Shan *et al.*, 2007). Thus, flavonoids are an important topic of herbal research. Different phytochemical preparations with high TFC have exhibited antimicrobial activity (Rauha *et al.*, 2000). Of course, only a few papers are available that shows correlation between TFC and antimicrobial activities. Flavonoids are also extensively studied for their antioxidant properties. Structure-activity relationship studies of flavonoids have showed importance of the number and location of the phenolic -OH groups for the antioxidant efficacy. A quantitative structure-activity relationship model was developed for correlating the antioxidant capacity of flavonoids with various physicochemical parameters. A positive correlation among the parameters directly indicates that flavonoids are the key factors that control the antibacterial and antioxidant activities of these extracts.

The structure–antioxidant activity relationship of flavonoids is mainly evaluated against different free radicals, but RP assay, which determines directly the reducing capacity of a compound, i.e. 'antioxidant power', has not been given much focus (Firuzi *et al.*, 2005). Studies using RP of flavonoids show that the *o*-dihydroxy structure in the B ring and the 3-hydroxy group and 2,3-double bond in the C ring give the highest contribution to the antioxidant activity. Thus, in the present study, RP was chosen instead of radical-scavenging activity and a good correlation was obtained with total flavonoid content as well as with the antibacterial potential.

From the presented data it is clear that the mere presence of flavonoids in extracts cannot be the sole determining factor for its activity; rather the structures of these compounds are also very important. The orientation of side chains and presence and number of hydroxyl group in the phenolic compounds is the major determining factor for antimicrobial and antioxidant capacity. It is also noteworthy that the action of each phenolic compound against different microbes is also very complicated (Kalemba and Kunicka, 2003). Thus, it is well understood that for better correlation, isolation of the compounds and identification of its structure would only establish its mechanism of action against microorganisms, since in crude extracts a large quantity of diverse polyphenol compounds, with varied potency are present.

The present study demonstrated that several of the extracts contained high levels of phenolics and possessed strong antibacterial and antioxidant activities. They could be a potential source for inhibitory substances against some foodborne bacterial pathogens as well as food spoilage bacteria.

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