

## A STUDY ON PHYTOCHEMICALS, ANTIMICROBIAL, AND SYNERGISTIC ANTIMICROBIAL ACTIVITIES OF *HIBISCUS SABDARIFFA*

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Received: 17 March 2018, Revised and Accepted: 08 March 2019

**ABSTRACT**

**Objective:** The aim of the present study was to screen phytochemical constituents and evaluate antimicrobial and synergistic antimicrobial properties of leaves and stem of *Hibiscus sabdariffa*.

**Methods:** The extraction was done by cold maceration method using 80% aqueous methanol. The antimicrobial efficacy and synergistic antimicrobial activity were carried out by disc diffusion assay against the bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

**Results:** Phytochemical analysis revealed the presence of carbohydrate, protein, alkaloids, phytosterols, flavonoids, and diterpenes in both the leaves and stem extracts while saponins, phenol, and tannins were found to be present only in the leaf extract. Both the extracts inhibited the tested bacteria with minimum inhibitory concentration value of 10 mg/ml. Aqueous methanolic extract of leaf showed higher antibacterial activity against *S. aureus* and *P. aeruginosa* and also exhibited synergistic activity with the antibiotic chloramphenicol against *S. aureus*.

**Conclusion:** The present study concludes that *H. sabdariffa* is a potential source of bioactive components and also provides information on synergistic activity of leaf extract. The results can contribute to the development of potent antibacterial agents.

**Keywords:** *Hibiscus sabdariffa*, Synergistic effect, Antimicrobial, Phytochemicals.

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**INTRODUCTION**

From time immemorial traditional culture in the world is associated with medicinal plants. These medicinal plants are exploited as a source of ingredients in traditional medicine. They contain vast array of phytoconstituents for combating chronic and infectious diseases and ailments. These phytoconstituents are probably secondary metabolites playing an integral role for the survival of plants including growth regulation, inter- and intra-specific interactions, and protection against predators and infection [1,2].

Infection and infectious agents exist from the beginning of life. Infectious diseases are still the world's leading cause of mortality. However, the mortality rate caused by pathogens has been decreasing slowly. While considering the statistics related to number of deaths annually from 1990-2010, it has been found out that there is a decline of only 1% per year. Reports of the World Health Organization have suggested that there will be 13 million deaths associated to these causes in 2050 [3].

For many decades, plants have been traditionally used as the source of antimicrobial substances. Ethnobotanical studies have revealed the importance of plants in treating infectious diseases. Some of the major antimicrobial compounds produced by plants include phenolics, polyphenols, terpenoids, essential oils, lectins, polypeptides, and alkaloids. Plant extracts containing these compounds provide an enhanced effect [4]. These compounds act on bacteria through different mechanisms: "Inactivation of proteins, adhesins, and enzymes, block cell-to-cell signaling pathways and quench production of virulence factors (e.g., exotoxins) [5,6], and disrupt or inhibit the formation of biofilms" [7-9].

*Hibiscus sabdariffa* L. (Family: Malvaceae), also known as Roselle or Sorrel, is native to Asia (India to Malaysia) or tropical Africa. The plant is widely distributed in India, Caribbean, Africa, Central America,

Brazil, Hawaii, Florida, Australia, and the Philippines. In Indian languages, it is known as Gongura, Lalambari, Patwa (Hindi), Lal-mista, Chukar (Bengali), Lal-ambadi (Marathi), Pulicha Keerai (Tamil), Yerra gogu (Telugu), Pulachakiri, Pundibija (Kannada), Polechi, Pulichchai (Malayalam), and Chukkiar (Assam) [11].

It is a dicotyledonous, annual or perennial herb or woody-based subshrub, growing 2-2.5 m tall. Different parts of this plant comprise vast and diverse phytochemicals. *H. sabdariffa* is enriched with quercetin, luteolin, chlorogenic acid, protocatechuic acid, pelargonic acid, sitosterol and ergosterol, hydroxycitric acid, delphinidin-3-sambubioside, and cyaniding-3-sambubioside [12]. In folk medicine, it is widely utilized as a remedy for various ailments such as cough, fever, hangover, debility, dyspepsia, bilious conditions abscess, heart diseases, hypertension, and neurosis [13,14]. The plant is reported to exhibit antioxidant, nephro- and hepato-protective, renal/diuretic effect, anticholesterol, antidiabetic, antihypertensive, antihyperuricemic and antiarthritic effects [15-17]. It modulates obesity through its antioxidant mechanism, and fat metabolism, namely reduction in adipogenesis, fat absorption, and excretion [18]. It is used to resist various intestinal infections added that *H. sabdariffa* flowers can be used in relaxing the pain of muscles of the uterus and intestine. Furthermore, it exhibits extensive antibacterial properties. Its purplish sepals (calyx and epicalyx) rich in anthocyanin are used economically in food (Jam and Jelly) and cosmetic industries as a source of natural coloring agent [19].

Despite the development made in understanding the microbes and progress of control measures, incidents of epidemics pose a serious public health issue. This is due to the advent of drug-resistant microorganisms and unknown microbial diseases. Resistance to drugs can be natural or acquired. Mutation changes or genetic combination are considered to be the cause of the resistance of microorganism to the antimicrobial drugs [10]. Thus, the unreceptiveness of microorganisms to the modern medicines and the safety and side effect issues drives

for the renewed interest for alternate medicine. Synergism finds a breakthrough attention to fight against microorganism, particularly resistant microorganisms. Further, in the current scenario, synergism among plant extracts and antibiotic is of renewed interest. Hence, in the present study, leaves and calyces of *H. sabdariffa* are screened for their phytochemical constituents and their antibacterial and synergistic antibacterial activities are evaluated.

## MATERIALS AND METHODS

### Collection of plant material

Plant was collected near the areas of Pallavaram, Chennai, India. The collected plant material was then authenticated by Dr. P. Jayaraman, Plant Anatomy Research Centre, Chennai, Tamil Nadu. The plant materials (leaf and stem) were then thoroughly washed with tap water and dried under shade. The dried plant parts were homogenized to a fine powder and used for extraction.

### Preparation of plant extract

About 10 g of each of the powdered samples were extracted with 80% aqueous methanol for 5 days. After filtration using cheesecloth, the filtrate was refiltered again using Whatman No.1 filter paper to remove fine residues in the filtrate. Methanol and water were evaporated while extracts were then obtained.

### Qualitative phytochemical analysis

Phytochemical analysis of *H. sabdariffa* was done with the leaf and stem extracts by following the standard procedures [20]. All the crude extracts were analyzed for the presence of alkaloids, carbohydrates, saponins, protein, phenols, diterpenes, steroids, flavonoids, glycosides, and tannins.

### Microbial samples

The test organisms include Gram-positive *Staphylococcus aureus* ATCC29213 and Gram-negative *Pseudomonas aeruginosa* ATCC15442. The bacteria were cultured in the nutrient broth 37°C and maintained on nutrient agar slants at 4°C.

### In vitro antibacterial activity

*In vitro* antibacterial activity was examined for aqueous methanolic extract of leaves and stem of *H. sabdariffa*. Antimicrobial activities of these extracts were evaluated by disc diffusion method [21]. For all the bacterial strains, overnight cultures grown in broth were adjusted to an inoculum size of  $2 \times 10^6$  colony-forming units (CFU)/ml with 0.5 McFarland standard and inoculated in the agar plates [22]. Petri plates were prepared with 15 ml of sterile Mueller-Hinton agar. The test culture (0.1% of a suspension containing  $10^6$  CFU/ml bacteria) was swabbed and allowed to dry for 10 min. Sterile 6 mm discs were impregnated each with appropriate extract of different concentrations 20 mg/ml, 40 mg/ml, 80 mg/ml, and 160 mg/ml. The loaded discs were placed on the surface of medium and the extract was allowed to diffuse for 5 min and the plates were kept for incubation at 37°C for 24 h. Reference antibiotic chloramphenicol of concentration 10 mg/ml was used.

### Minimum inhibitory concentration (MIC)

MIC is "the least concentration of an antimicrobial that will hinder the visible growth of a microorganism after overnight incubation [23]." For determining MIC, a modified dilution method was performed [24]. Each extract was diluted into various concentrations of 10, 8, 6, 4, and 2 mg/ml in sterile nutrient broth in test tubes. Using standard wire loop, a loopful (10  $\mu$ l) of *P. aeruginosa*, 0.5 McFarland standard, was inoculated into test tubes containing 1 ml of the various concentrations of the appropriate extracts in nutrient broth. Similarly, this was repeated for *S. aureus*. Incubation of the tubes was carried out at 37°C for 18–24 h, and thereafter, growth or turbidity was observed.

### Evaluation of the synergistic effect

Synergistic antimicrobial activity was done using disc diffusion method with modifications. Agar plates were inoculated with the tested

microorganisms. Extract showing maximum antibacterial activity was taken for the study of synergistic activity. Sterile 6 mm discs were impregnated with appropriate extract of the concentration of 20–160 mg and antibiotic of the concentration of 10 mg/ml and allowed to saturate for 30 min and are placed on the agar plates. All the plates were then subjected to incubation at 37°C for 24 h. Zone of inhibition (ZOI) that appeared around the discs was measured and recorded.

$$\text{Synergistic effect \%} = (B-A)/A \times 100$$

Where, A is ZOI for the antibiotic and B is ZOI for the antibiotic+the plant extract [25,26].

### Statistical analysis

The experimental results were expressed as mean  $\pm$  standard error of triplicates.

## RESULTS AND DISCUSSION

The present work attempt to establish *in vitro* synergy between aqueous methanolic extract of the medicinal plant and commonly used antibiotic (chloramphenicol) emphasizing the potential role of phytochemicals in increasing the effectiveness of antibiotics.

### Preliminary phytochemical analysis

The leaves and stem aqueous methanolic extracts of *H. sabdariffa* were subjected to preliminary phytochemical analysis. The analysis indicated the presence of carbohydrate, protein, alkaloids, phytosterols, flavonoids, and diterpenes in both the leaves and stem extracts, whereas saponins, phenol, and tannins were found to be present only in the leaf extract. Alkaloids, saponins, tannins, flavonoids, phenols, and several other aromatics have the capability to resist microbial invasion [27]. It is documented that the chemical structure of the phytochemicals plays an important role in determining antibacterial activity. For instance, flavonoids which are hydroxylated phenolic substance are synthesized by plants in response to microbial infection. They complex with extracellular and soluble proteins and also with bacterial cell wall [4]. Saponins exhibit antibacterial activity by causing leakage of bacterial protein and enzymes [28]. Its biological activity depends on its aglycone part or number of sugar residues [36]. Similarly, basic character of tannins enables it to bind with proteins and damage bacterial cell membrane. Tannins also hinder microbial growth by precipitating microbial protein and make nutritional proteins unavailable [29]. Thus, *H. sabdariffa* has been found to be the reservoir of phytochemicals and capable of exhibiting antibacterial activity against number of diseases. The overall result is tabulated in Table 1.

### Evaluation of antibacterial activity

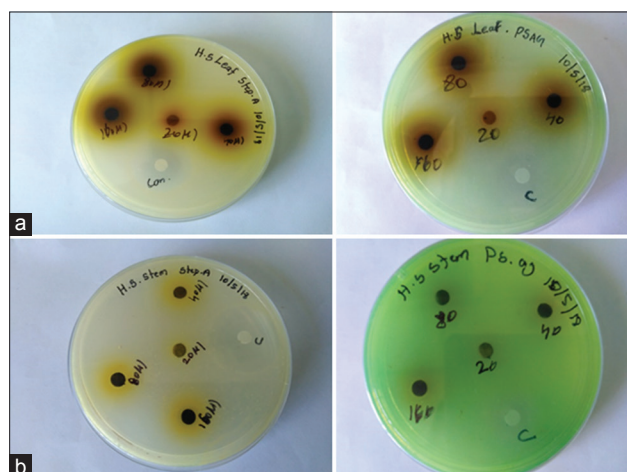
*S. aureus* and *P. aeruginosa* were tested to evaluate the antibacterial activity of *H. sabdariffa*. Different parts of *H. sabdariffa* showed variable antibacterial potential and they responded to the tested bacteria in a varied manner (Fig. 1). The leaf and stem extracts of *H. sabdariffa*

**Table 1: Phytochemical analysis of *H. sabdariffa* leaves and stem extracts**

S. No.	Phytochemical	<i>H. sabdariffa</i>	
		Leaf	Stem
1	Alkaloids	+	+
2	Carbohydrates	+	+
3	Saponins	+	-
4	Phytosterols	+	+
5	Phenols	+	-
6	Tannins	+	-
7	Flavonoids	+	+
8	Proteins and amino acids	+	+
9	Diterpenes	+	+
10	Glycosides	-	-

+: Presence, -: Absence, *H. sabdariffa*: *Hibiscus sabdariffa*

inhibited the tested bacteria with MIC value of 10 mg/ml. The result of this investigation revealed that at different concentration (20–160 mg/ml) leaves of *H. sabdariffa* were effective showing higher inhibitory actions against *S. aureus* and *P. aeruginosa* than the stem extract (Table 2). The bacteria responded to stem extract only at higher concentration (80 and 160 mg/ml) (Table 3). Meanwhile, leaves of



**Fig. 1: Antibacterial activity of the aqueous methanolic extract of *Hibiscus sabdariffa*, (a) leaf, (b) stem**

Sa: *Staphylococcus aureus*, Pa: *Pseudomonas aeruginosa*

**Table 2: Antibacterial activity of leaves and stem extracts of *Hibiscus sabdariffa***

Conc. mg/ml	Inhibition zone (mm)	
	<i>S. aureus</i>	<i>P. aeruginosa</i>
20	5±0.4	6±0.08
40	8±0.23	11±0.56
80	10±0.45	11±1.00
160	11±1.58	12.33±0.56
Control (10 mg/ml)	20±0.00	10±0.47

Values are expressed as Mean±SE, Control: Chloramphenicol, *S. aureus*: *Staphylococcus aureus*, *P. aeruginosa*: *Pseudomonas aeruginosa*

**Table 3: Antibacterial activity of aqueous methanolic extract of the stem of *Hibiscus sabdariffa***

Conc. mg/ml	Inhibition zone (mm)	
	<i>S. aureus</i>	<i>P. aeruginosa</i>
20	-	-
40	-	-
80	5±0.18	-
160	9±0.12	6±0.14
Control (10 mg/ml)	26±0.14	11±0.12

Values are expressed as Mean±SE, Control: Chloramphenicol, *P. aeruginosa*: *Pseudomonas aeruginosa*, *S. aureus*: *Staphylococcus aureus*

**Table 4: Synergistic antibacterial activity of aqueous methanolic extract of the leaves of *Hibiscus sabdariffa***

Zone of inhibition	Extract+antibiotic (mg/ml)				Chloramphenicol (mg/ml)
	20	40	80	160	
<i>S. aureus</i>	16±0.2	20±0.4	20±0.4	23±0.4	20±0.3
Combination effects	A	I	I	S (64%)	
<i>P. aeruginosa</i>	-	-	-	10±0.2	10±0.4
Combination effects	-	-	-	I	

Values are expressed as Mean±SE, Antibiotic: Chloramphenicol, S: Synergism, I: Identical, *S. aureus*: *Staphylococcus aureus*, *P. aeruginosa*: *Pseudomonas aeruginosa*

*H. sabdariffa* demonstrated antibacterial activity in a dose-dependent manner. This observation is in agreement with a study in which leaves of *H. sabdariffa* were found to inhibit Gram-positive bacteria: *Bacillus cereus*, *Micrococcus luteus*, and *S. aureus* and Gram-negative bacteria: *Escherichia coli*, *P. aeruginosa*, and *Salmonella choleraesuis* [31]. In another study, aqueous extract of root and calyx of *H. sabdariffa* demonstrated marked antibacterial activity than that of stem [32]. The potent antibacterial properties of the leaves of *H. sabdariffa* could be attributed to its richness in phytochemicals. Studies are successful in isolating proteins from plant extracts and demonstrating its efficiency as antimicrobial agent [30]. Our phytochemical studies revealed the presence of alkaloids, phenolic compounds, flavonoids, and saponins. These compounds are considered to be the major groups of antimicrobial compounds in plants.

#### Synergistic antibacterial activity

Synergistic/additive interactions occur as a result of a combined effect of active compounds from extracts and antibiotics. Phytochemicals play an important role in dealing synergism as they have an impact on growth and metabolism of microorganisms [4].

The aqueous methanolic extract of the leaves of *H. sabdariffa* which reported to have higher antibacterial activity has been chosen for synergistic study and the results are shown in Table 4.

Synergistic activity of *H. sabdariffa* leaves and antibiotic chloramphenicol was carried out to find the effectiveness against *S. aureus* and *P. aeruginosa*. It was evident that the above-said combination exhibited an identical effect with *P. aeruginosa* but synergistic effect with *S. aureus* at higher concentration (160 mg/ml). Such synergistic effect was observed with aqueous extract of *H. sabdariffa* calyces and the antibiotic clarithromycin, amoxicillin, or metronidazole against *Helicobacter pylori* strains [33]. However, in one another study, *H. sabdariffa* calyces exhibit synergistic effect against fungal strains but not with bacteria [34]. Several studies have reported synergistic activity at higher concentration. For example, when lemon balm was studied with ampicillin for synergism at a concentration range of 5–400 mg/ml, it was found that it is susceptible to *Klebsiella pneumonia* at 200 mg/ml [35]. Such a combination therapy can be effective as it increases the efficacy of antibiotic by either blocking or circumventing resistance mechanisms.

#### CONCLUSION

Nowadays, renewed interest has been developed to explore alternate treatment to reduce the dose of antibiotics, particularly combination of plant extract and synthetic antibacterial agents. Apparently, low or non-toxicity of plant preparations and their low cost as compared to synthetic drugs envisage its use as antibacterial agents. Based on the results, it is concluded that antibacterial activity of aqueous methanolic extract of the leaves of *H. sabdariffa* is found to be effective against both the tested bacteria and exhibits synergism along with antibiotic, chloramphenicol against *S. aureus*. Thus, the combination of natural antibacterial agent and antibiotic will pave a way for alternative treatment for infections. In future, more insight has to be carried out to confirm these activities as well as the mechanisms of action *in vivo*.

## AUTHORS' CONTRIBUTIONS

All authors have equal contribution in bringing out this article.

## CONFLICTS OF INTEREST

None.

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