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Full Length Research Paper

Inhibitory activity and phytochemical assessment of ethno-medicinal plants against some human pathogenic bacteria

Sumana Das[1]*, Kuntal Das[2] and Veenapani Dubey[1]

[1]Department of Botany, C.M.D. P.G. College, Bilaspur-495001, Chhattisgarh, India.
[2]Department of Botany, Government. P.G. Science College, Bilaspur-495001, Chhattisgarh, India.

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The present investigations evaluate the inhibitory efficacy of different solvent extracts prepared from seven ethno-medicinal plant species against four human pathogenic bacteria. Antibacterial activity, tested by agar well diffusion method revealed that, *Boerhaavia diffusa* and *Andrographis paniculata* showed maximum inhibitory activity on pathogenic bacteria comparable with streptocycline efficacy. The most susceptible pathogen in the present investigation was found to be *Klebsiella pneumoneae* followed by *Staphylococcus aureus* while *Pseudomonas fluorescence* was the most resistant. Afterwards, in qualitative phytochemical analysis alkaloids, tannins and flavonoids were commonly found as majority from tested plant species. Further, thin layer chromatography detected recurrently occurring flavonoidal compounds, namely Apigenin and Chrysoeriol in different plant species.

Key words: Ethno-medicinal plants, human pathogen, bacteria, phytochemical, thin layer chromatography.

INTRODUCTION

Historically, plants have provided a good source of antimicrobial agents which are highly effective in the fight against human infections (Cowan, 1999). Infectious diseases are serious health problem worldwide. Commercial antimicrobial drugs used haphazardly in the treatment of many infectious diseases have inevitably led multiple drug resistance (MDR) in human pathogenic microorganisms (Sokmen et al., 2004). Even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased (Das et al., 2010). Considering these there is an urgent need to search for alternative method for the management of bacteria or fungi without any toxicity to the consumer and is eco-friendly.

In the last few years, a number of studies have been conducted in different countries to prove antimicrobial efficacy of botanicals (Sharma and Kumar, 2009; Thenmozhi and Rajeshwari, 2010). Plant-derived substancesespecially from ethno-medicinally important species have recently become of great interest owing to their versatile applications (Baris et al., 2006). Medicinal plant contains substances that can be used for therapeutic purpose or which are precursors for the synthesis of useful drugs (Sofowara, 1982). Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Hammer et al., 1999). The medicinal value of plants lies in some chemical substances. The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds (Duraipandiyan et al., 2006; Edeoga et al., 2005).

Chhattisgarh placed in deccan bio-geographical area of India is one of the largest tribal state. The state is declared as an 'Herbal State' and houses a rich and unique biological diversity of several important medicinal plant species which are used by local tribes more often. Thus considering vast potentiality of ethno-medicinal plants as a source of new chemotherapeutic and antimicrobial agents, detailed investigations was conducted to test the efficacy of some species against some human pathogenic bacteria.

*Corresponding author. E-mail: sumanarsin@rediffmail.com.

Table 1. Characteristics details and uses of studied plants.

Plants	Family	Local name*	Parts used	Ethno-medicinal use
Andrographis paniculata	Acanthaceae	Kalmegh	Leaf	Antihepatotoxic, antimalarial, antihepatitic, antithrombogenic, antiinflamatory and antipyretic.
Bryophyullum calycinum	Crasulaceae	Patharchatta	Leaf	Antibacterial, antifungal, antiinsecticidal.
Boerhaavia diffusa	Nyctaginaceae	Punarnava	Leaf	Leucorrhea, root juice in treating asthama, scanty urine, and heumatism, and stomachache, of dyspepsia, jaundice, enlargement of spleen, abdominal pain, abdominal tumors.
Cissus quadrangularis	Vitaceae	Hadjod	Stem	Osteoporosis, asthma, cough, hemarrhoids, and gonorrhea.
Euphorbia hirta	Euphorbiaceae	Dudhi	Leaf	Asthma and respiratory tract inflammations, coughs, chronic bronchitis and other pulmonary disorders.
Plumbago zeylanica	Plumbaginaceae	Chittrak	Leaf	Abortifacient, antiperiodic, appetizer, astringent, digestive stimulant, sudorific and used weakness after fever.
Vitex negundo	Verbanaceae	Nirgundi	Leaf	Applied to rheumatic swellings of the joints and sprains, antinflammatory, anti-bacterial, anti-fungal and analgesic activities, leaf smoke repels mosquitoes,

* In India.

MATERIALS AND METHODS

Plant materials

Medicinal plant species used for this study were collected from the natural habitat of Chhattisgarh state (17° to 23° N latitude and 80°4' to 83°38' E longitude). Plant sp ecies were identified using herbarium in the Department of Botany, C.M.D. Post Graduate College, Bilaspur (Chhattisgarh). Seven important plant species of ethno-medicinal values were considered which are documented in Table 1 (Nair, 1998; Sala, 1994). Disease free fresh plant materials were used for preparation of extracts.

Preparation of plant extracts

Fresh leaves were thoroughly washed several times under running tap water followed by surface sterilization by mercuric chloride (0.01%) (Nahunnaro, 2008). Plant materials were then chopped in a kitchen blender. Extraction was done using the method described by Joseph et al. (2008). For the preparation of hot water extract, macerated plant material were immersed in required quantity of water (1:1 w/v) and boiled for 30 to 40 min whereas, for extraction with cold water, methanol, ethanol, butanol and acetone the macerated plant materials were dipped in required quantity of solvents (1:1 w/v) for 5 to 6 h. The crude extracts were then strained through double-layered muslin cloth and centrifuged at 5000 rpm for 5 min (Priya and Ganjewala, 2007). The supernatant was collected and evaporated to remove the respective solvents and thus 100% stock solution was obtained which was stored in refrigerator for further use.

Bacterial pathogen

Five human pathogenic bacteria *viz. Escherichia coli, Pseudomonas fluroscence, Staphylococcus aureus*, *Klebsiella pneumoniae* and *Bascillus subtilis* were obtained from the Department of Microbiology, Chhattisgarh Institute of Medical Sciences (C.I.M.S.), Bilaspur, Chhattisgarh and were maintained as stock cultures in nutrient broth.

Antibacterial activity

To evaluate the antibacterial activity of plant extracts *in vitro*, 'well in agar' method was applied (Onkar and Dhingra, 1995). Bacterial inoculum size was standardized following the procedure as described by Andrews (2001). In brief, 24 h old single colony of bacteria was dissolved in 5 ml of Muller Hinton broth. The bacterial suspension was adjusted by supplementing Muller Hinton broth to match the density of 0.5 McFarland standards (McFarland, 1907). The suspension thus obtained contained approximately 10^8 cfu/ml (colony forming units/ ml) which served as inoculum.

100 ml of Nutrient Agar Base medium (NAB) was prepared in 250 ml Erlenmeyer flask and sterilized in

autoclave. Using a micro-pipette, an aliquot of 0.1 ml of inoculum was aseptically added to sterilized semisolid media at a temperature of 40 to 45℃. The flasks were swirled manually to homogenize the inoculum to the media and dispensed in Petri plates (approximately 25 ml in each plate). After solidification a hole was punched at the center using a sterile cork borer of 7 mm diameter (Bradshaw, 1992).

Plant extracts were used at 100% concentration for anti-bacterial assay. Using a micropipette, 0.1 ml of plant extract was dispensed aseptically in the well. Media with same volume of sterile distilled water was served as control whereas media with Streptocycline (100 ppm) served as antibiotic control. The treated Petri plates were incubated at 37±1℃ for 24 h (McCuen and McCuen, 1988; Collins et al., 1989). Diameter of inhibition zone formed around the well was measured twice perpendicularly, using a transparent ruler. All the extracts were tested in triplicates and calculations were carried in triplicate with mean values (Gupta et al., 1962).

Phytochemical assessment

Phytochemical tests were carried out on the aqueous extract and on the powdered plant materials using standard procedures as described by Sofowara (1993), Trease and Evans (1978), Harborne (1973) and Edeoga et al. (2005).

Isolation of active constituents from plant extracts by TLC system

Thin layer chromatography (T.L.C.) technique of different crude extracts was applied for the detection of flavonoids in B.A.W. (Butanol: Acetic Acid: Water, 4: 1: 5) medium (Stahl, 1969).

RESULTS AND DISCUSSION

Antibacterial activity

Analysis of the data in Table 2 revealed that ethanolic and methanolic extract of *Andrographis paniculata* was most effective against *K. pneumoniae* showing inhibitory zone of 26.1 and 25.3 mm, respectively. The finding in present case may further be explored for bioactive compound which is responsible for the antibacterial activity. So far there have been no reports of this plant to be used as antimicrobial. Similarly, *K. pneumoniae* was greatly inhibited by methanolic leaf extracts of *Euphorbia hirta* (25.1 mm) as well as *Boerhaavia diffusa* (25.1 mm) and ethanolic extract of *Vitex negundo* (25.4 mm) whereas, these extracts were comparatively less effective against *S. aureus*, *P. fluorescence* and *E. coli*. Hot water extract of *Bryophyllum calycinum* responded most to the *E. coli* in terms of inhibition zone that is, 23.3 mm followed by *K. pneumoniae* (19.2 mm). A significant zone of inhibition was also observed against *S. aureus* (21 mm) with cold water extract from same plant. The positive control used by Streptocycline showed an inhibition zone of 32.5 mm irrespective of the test organisms. Thus, in terms of sensitivity to the bacterial stains these may be arranged as *K. pneumoniae* > *S. aureus* > *E. coli* > *P. fluorescence*.

It is quite evident that the extract of *V. negundo* has more influence in inhibition of bacterial growth compared to *Cissus quadrangularis*, particularly against *K. pneumoniae*. However, *C. quadrangularis* stem was found to be least antibacterial irrespective of bacterial pathogen. This fact can be correlated with its indigenous use as a bone setting material. Hot water leaf extract of *Plumbago zeylanica* (leaf extract) was effective against *S. aureus* and showed an inhibition zone of 30.1 mm which was at par with standard antibiotic control of Streptocycline (32.5 mm). It is justifiable to note here that *S. aureus* is a wide-spread parasite, occurring on human skin and mucus membranes. Ahmad and Beg (2001) have also found antimicrobial properties of *P. zeylanica* (roots) against *S. aureus*, showing a zone of 10-20 mm inhibition. Earlier, Kannan et al. (2009) have reported 70% growth inhibition against *B. subtilis* and *S. aureus* with *Terminalia chebula* fruit ethanolic extract. Several other workers have also reported antibacterial activity of other plants, like, *Nyctanthes arbortristis* (ethanolic leaf extract) against *E. coli*, *K. penumoniae*, *S. aureus* and *B. subtilis* (Sathiya et al., 2008). In terms of antibacterial activity the medicinal plants may be arranged as - *B. diffusa* > *A. paniculata* > *P. zeylanica* > *V. negundo* > *B. calycinum* > *C. quadrangularis* > *E. hirta* (Figure 1).

Phytochemical analysis

A wide range of phytochemicals, such as, alkaloids, tannins and flavonoids were commonly found in almost all plant species investigated (Table 3). All the major phyto-chemical groups, namely alkaloid, flavonoid, tannin, saponin, steroid, terpenoid and cardiac glycosides were present in *A. paniculata*. However, Anthraquinone and phlobatannin were absent in the plant. Other phytochemical groups such as the presence of tannin, steroid and cardiac glycosides were detected in the leaf extracts of *B. diffusa*; whereas, rest of the phytochemical groups, namely anthraquinone, alkaloid, flavonoid etc. were not detected in the plant. However, both *E. hirta* and *B. calycinum* did not show the presence of anthraquinone, phlobatannin, cardiac glycosides and terprenoids in the extracts. Flavonoids were detected by *E. hirta* and *B. calycinum*. Alkaloid, tannin, saponin and steroid were detected in the leaves of *E. hirta*; while *B. calycinum* did not show the presence of these phytochemical groups. *P. zeylanica* showed the presence of anthraquinone along with tannin, saponin, flavonoid and cardiac glycoside. Alkaloid, steroid, phlobatannin and mterprnoid were not present in the extracts of *P. zeylanica*. Only flavonoids were detected in *C. quadrangularis*; whereas, rest of the phytochemical groups was absent. *V. negundo* confirmed the presence of alkaloids, steroids, flavonoids, cardiac glycosides and terpenoids. Other phytochemical compounds, such as, saponins, tannins and phlobatannins were absent in *V. negundo*. Fokunang et al. (2000) suggested that antifungal activity of solvent

Table 2. Antibacterial activity of different medicinal plant species as expressed by inhibition zone 48 h. after incubation of four human pathogenic bacteria.

Organisms	Inhibition Zone (mm) ± S.D.					
	Plant*	Cold water	Hot water	Methanol	Ethanol	Mean
Escherichia coli	*Bc*	10.4±0.34	23.3±0.64	18.4±0.28	15.1±0.33	17.4
	Ap	16.2±0.67	24.3±0.14	20.5±0.84	0±0.00	15.3
	Eh	0±0.00	16±0.44	20.3±0.23	19.4±0.23	13.9
	Bd	13.1±0.43	14.2±0.75	15.4±0.22	19.1±0.55	15.5
	Vn	0±0.00	0±0.00	8.2±0.34	20.1±0.13	7.1
	Cq	11.1±0.34	15.3±0.84	12.4±0.56	13.2±0.33	13
	Pz	0±0.00	0±0.00	18.2±0.11	14.3±0.44	8.1
Klebsiella pneumoniae	*Bc*	0 ±0.00	19.2±0.38	14.4±0.43	11.2±0.27	11.2
	Ap	0±0.00	11.4±0.36	25.3±0.59	26.1±0.67	15.7
	Eh	0±0.00	23.2±0.34	25.1±0.56	22.1±0.27	17.6
	Bd	20.2±0.73	20.4±0.23	19.1±0.88	22.1±0.45	20.5
	Vn	0±0.00	20.1±0.55	19.5±0.46	25.4±0.55	16.3
	Cq	10.2±0.56	0±0.00	12.1±0.78	0±0.00	5.6
	Pz	0±0.00	15.4±0.02	20.3±0.11	26.1±0.23	15.5
Pseudomonas fluorescence	*Bc*	16.1±0.31	15.4±0.72	0±0.00	7.3±0.35	9.7
	Ap	21.4±0.84	0±0.00	17.3±0.24	0±0.00	9.7
	Eh	19.3±0.35	0±0.00	0±0.00	0±0.00	4.8
	Bd	8.1±0.75	17.0±0.65	16.3±0.33	12.0±0.35	13.4
	Vn	0±0.00	11.2±0.67	20.1±0.24	24.3±0.56	13.9
	Cq	0±0.24	0±0.00	18.2±0.56	23.3±0.44	10.4
	Pz	0±0.00	0±0.00	16.5±0.11	0±0.00	4.1
Staphylococcus aureus	*Bc*	21±0.23	11.5±0.12	0±0.00	10.4±0.34	10.7
	Ap	0±0.00	22.1±0.54	20.4±0.84	19.1±0.77	15.4
	Eh	0±0.00	0±0.00	7.1±0.66	16.5±0.83	5.9
	Bd	19.2±0.67	0±0.00	8.2±0.43	0±0.00	6.9
	Vn	0±0.00	22.3±0.55	18.2±0.75	18.4±0.78	14.7
	Cq	15.2±0.45	0±0.00	19.4±0.24	24.3±0.23	14.7
	Pz	27.2±0.23	30.1±0.55	22.4±0.11	25.3±0.71	26.3

Antibiotic Streptocycline at 300 ppm was used as positive control for bacteria with mean inhibition zone of 32.5±0.36. *Plant = *Bc* - *B. calycinum*; *Ap* - *A. paniculata*; *Eh* - *E. hirta*; *Bd* - *B. diffusa*; *Vn* - *V. negundo*; *Cq* - *C. quadrangularis*; *Pz* - *P. zeylanica.*

based plant extracts depends on the nature and amount of active phytochemicals present in it.

Thin layer chromatography (T.L.C.)

Flavonoid assessment by thin layer chromatography (TLC) of different medicinal plant extracts prepared in various solvents is elaborately depicted in Table 4. The data clearly indicates that there is an array of flavonoidal constituents present in the tested plant species. However, a close scrutiny of the results revealed that recurrently occurring flavonoidal compound is Apigenin followed by Chrysoeriol whereas, Tricin, Myrecetin and Gossypetin occurred less frequently (Figure 2). An antimicrobial property of flavonoid was also reported by Alan and Miller (1996).

C. quadrangularis posses maximum diversity in terms of flavonoidal compounds whereas, *V. negundo* detected with single compound Apigenin (Figure 2). Moreover, among the different plant extracts flavonoidal compound which found repeatedly are namely; Chrysoeriol in *A. paniculata*, Apigenin in *P. zeylanica*, Azaleatin in *E. hirta*, Apigenin in *V. negundo*, Apigenin and Isovitexin in *C. quadragularis*, and Quercetin and Isovitexin in *B. calycinim* (Figure 2).

The present investigation is an important step in developing plant based drugs which are eco-friendly for

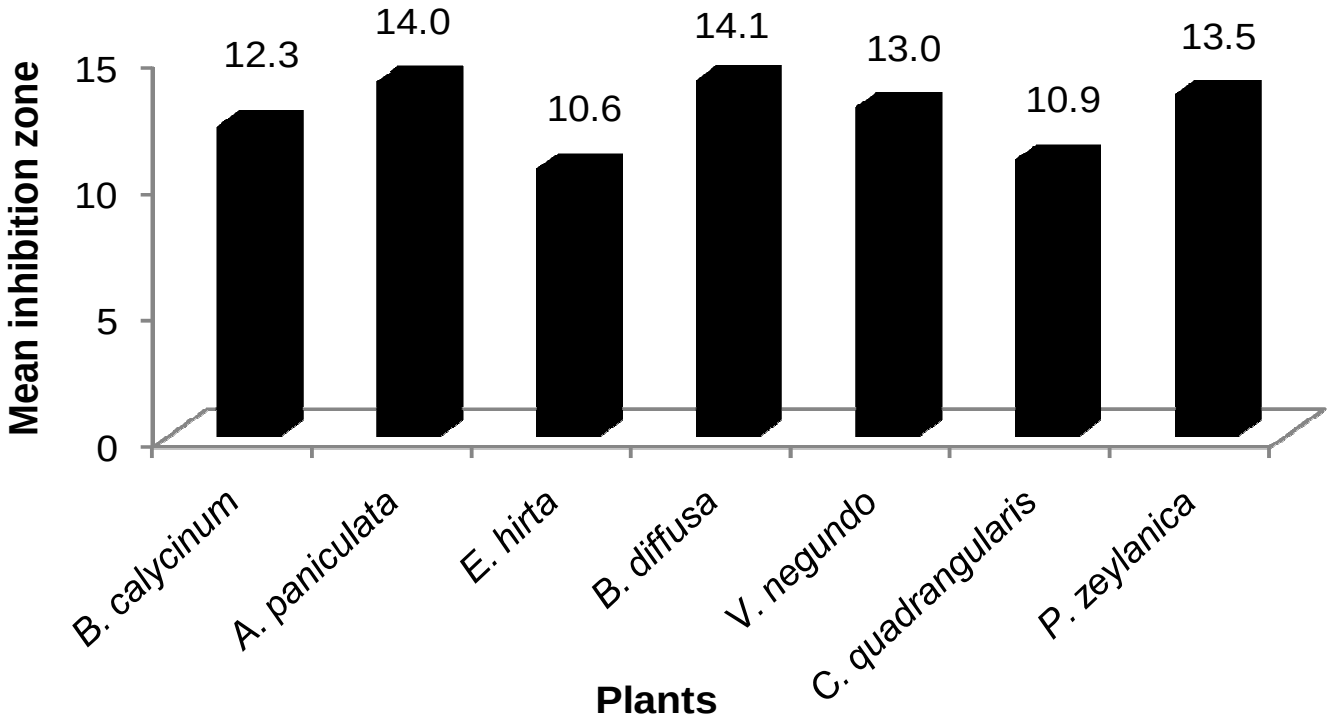

Figure 1. Inhibitory activity of different plant species as expressed by mean zone of inhibition irrespective of solvents extracts against pathogenic bacteria.

Table 3. Qualitative analysis of major phytochemical groups of tested medicinal plants.

Plants	Phytochemical groups								
	Anthra-quinone	Alkaloid	Tanin	Saponin	Steroid	Phlobatanin	Flavonoid	Cardiac glycoside	Terpenoid
Andrographis paniculata	-	+	+	+	+	-	+	+	+
Boerhaavia diffusa	-	-	+	-	+	-	-	+	-
Euphorbia hirta	-	+	+	+	+	-	+	-	-
Bryophyullum calycinum	-	-	-	-	-	-	+	-	-
Plumbago zeylanica	+	-	+	+	-	-	+	+	-
Cissus quadrangularis	-	-	-	-	-	-	+	-	-
Vitex negundo	-	+	-	-	+	-	+	+	+

+ = Present; - = Absent.

Table 4. Thin layer Chromatography of plant extracts for detection of flavonoidal compounds.

Plant species	Colour in				
	Extract	Rf x 100	Normal light	UV	Compound
Andrographis paniculata	Cold water	41.1	DB	DO	Vitexin
		88.2	YG	BrY	Kaempferol
	Hot water	40	DB	DO	Vitexin
		82.3	FG	YG	Chrysoeriol
	Ethanol	40.5	PG	YG	Isoorientin
		82.3	FG	YG	Chrysoeriol
	Methanol	41.1	PG	YG	Isoorientin
		82.3	FG	YG	Chrysoeriol
Plumbago zeylanica	Cold water	88	YG	FG	Apigenin
	Hot water	86	YG	FG	Chrysoeriol
	Ethanol	88	YG	FG	Apigenin
	Methanol	66	Y	BrY	Quercetin
		88	YG	FG	Apigenin
Euphorbia hirta	Cold water	47.8	PY	FY	Azaleatin
	Hot water	82.6	PY	BrY	Kaempferol
	Ethanol	47.8	PY	FY	Azaleatin
	Methanol	88.6	GY	YG	Apigenin
Vitex negundo	Cold water	89.5	GY	YG	Apigenin
	Hot water	89.5	GY	YG	Apigenin
	Ethanol	89.5	GY	YG	Apigenin
	Methanol	89.5	GY	YG	Apigenin
Cissus quadrangularis	Cold water	43.3	PY	BrY	Myricetin
		80	YG	YG	Chrysoeriol
	Hot water	56.6	PY	BrY	Isovitexin
		73.3	FY	YG	Tricin
	Ethanol	56.6	PY	BrY	Isovitexin
		91	PY	BrY	Apigenin
	Methanol	31.3	OB	DB	Gossypetin
		91	PY	BrY	Apigenin
Bryophyllum calycinum	Cold water	64.8	YG	BrY	Quercetin
	Hot water	64.8	YG	BrY	Quercetin
	Ethanol	56	PY	YG	Isovitexin
	Methanol	56	PY	YG	Isovitexin

FG = Fluorescent green; PY = Pale yellow; BrY = Bright yellow; DO = Dark ochre; YG = Yellow green; OB = Ochre brown; Y = Yellow; GY = Greenish yellow; FY = Fluorescent yellow; PG = Pale green.

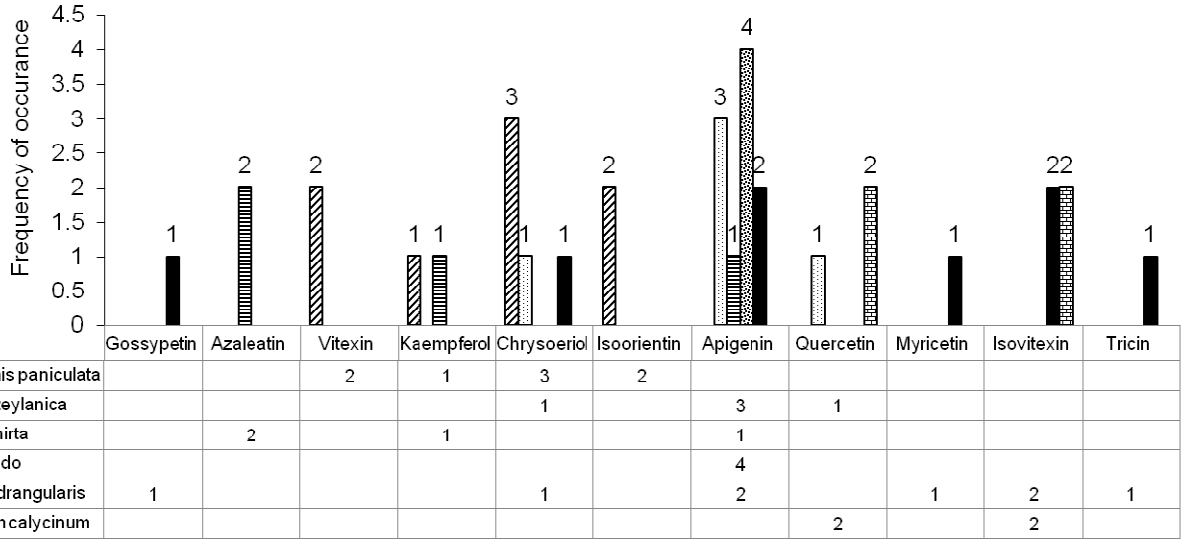

Figure 2. Frequency of occurrence of flavonoidal compounds in various plant extracts.

the control of human pathogenic bacteria and development of commercial formulation of botanicals. Further investigations are necessary in this direction based on field, animal trails and toxicological experiments.

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