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Antioxidant and antibacterial activity of flowers of *Calycopteris floribunda* (Roxb.) Poiret, *Humboldtia brunonis* Wall and *Kydia calycina* Roxb

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Abstract

The present study was carried out to determine antioxidant and antibacterial activity of solvent extracts of flowers of *Calycopteris floribunda* (Roxb.) Poiret (Combretaceae), *Humboldtia brunonis* Wall (Caesalpinaceae) and *Kydia calycina* Roxb (Malvaceae). The powdered flowers were extracted successively with petroleum ether, chloroform and methanol based on increased solvent polarity. Antibacterial activity of solvent extracts was performed against two Gram positive and two Gram negative bacteria by Agar well diffusion method. Antioxidant efficacy of solvent extracts was determined by DPPH free radical scavenging assay and Ferric reducing assay. Total phenolic and flavonoid contents were estimated by Folin-Ciocalteau reagent and Aluminium chloride colorimetric estimation method respectively. Maximum extract yield was obtained with methanol followed by chloroform and petroleum ether. Methanol extract of *C. floribunda* exhibited stronger antibacterial activity followed by *H. brunonis* and *K. calycina*. The flower extracts have shown dose dependent scavenging of DPPH free radicals. Scavenging activity was recorded highest in *H. brunonis* followed by *C. floribunda* and *K. calycina*. Methanol extracts have shown remarkable scavenging potential followed by chloroform and petroleum ether extracts. In ferric reducing assay also, methanol extracts exhibited stronger reducing power than other solvent extracts. Methanol extract of *C. floribunda* showed reducing potential greater than that of ascorbic acid. Total phenolic and flavonoid contents were also high in methanol extracts of flowers and these contents were high in *H. brunonis*. A marked antibacterial and antioxidant activity of flower extracts was observed in this study. A positive correlation was observed between antioxidant activity and total phenolic and flavonoid contents of solvent extracts. However, such correlation was not observed in antibacterial activity. The presence of secondary metabolites such as flavonoids and phenolic compounds might be related to the antibacterial and antioxidant activity of flower extracts. These flowers can be used to prevent oxidative damage and to treat infectious diseases. Further, isolation of active principles and determination of their efficacies are under progress.

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INTRODUCTION

There exists a well maintained balance between antioxidant mechanisms and free radical generation in a normal healthy individual. This balance,

however, shift towards the production of excessive free radicals or deficit in antioxidant defence in diseased states and leads to a condition called oxidative stress. This oxidative stress is implicated in hundreds of diseases or disorders such as diabetes, cardiovascular diseases, neurological disorders such as Alzheimer's disease and Parkinson's disease, cancer and others. Antioxidants are substances which, when present at low concentrations, in relation to oxidizable substrates, significantly inhibit or delay oxidative processes. Endogenous antioxidants such as ascorbic acid, tocopherols, glutathione, uric acid, thiols etc., and antioxidant enzymes such as superoxide dismutase and catalase defend the body against oxidative stress. However, in pathophysiological conditions, there is an extra requirement for antioxidants from external sources especially food and medicinal plants [1-5].

Phenolic compounds are a large and diverse group of plant metabolites and are known to possess a wide array of biological activities including antioxidant activity. They are widely distributed in fruits, vegetables and cereals. Phenolic compounds have strong antioxidant activity both in vitro and in vivo and the activity is associated with their ability to scavenge free radicals, break radical chain reactions and chelate metal ions. Increased consumption of phenolic compounds is found to be associated with reduced risk of several diseases such as cardiovascular diseases and certain types of cancer [6-10]. Flavonoids are a group of plant polyphenols. They constitute a large group of natural occurring plant phenolic compounds and include flavones, flavonols, isoflavones, flavonones and chalcones. They contain a characteristic C6-C3-C6 structure with free hydroxyl groups attached to aromatic rings. They are known to possess diverse biological activities including antioxidant activity. The antioxidant property of flavonoids is due to scavenging free radicals or by other mechanisms such as singlet oxygen quenching, metal chelation and lipoxygenase inhibition [8,10,11].

The primary and secondary metabolism of plants, animals and microorganisms results in products that have several applications in pharmacy, medicine and other fields. The discovery of antibiotics is one of the most significant health-related events of modern times. Antibiotics have revolutionized the field of medicine in many respects and their discovery and subsequent use saved countless lives. However, situations like overuse and abuse of antibiotics led to the development of resistance among microorganisms. Microorganisms such as *Staphylococcus aureus*, *Pseudomonas aeruginosa, Mycobacterium tuberculosis* and others have developed resistance against a wide range of antibiotics. Hence, there is a constant need for the development of new antimicrobials from natural sources, particularly from plants. Plants produce a variety of substances and most are secondary metabolites which serve in defense against predators, responsible for typical odors and characteristic pigmented nature of plants. Most of the phytochemicals are extensively used as medicinal compounds for treatment of various ailments all over the world especially in under-developing and developing countries [12-14].

Flowers are one of the important parts of the plants and are known to contain a wide variety of phytochemicals. Many edible flowers have been used in food, food garnishing etc. in various parts of the world for centuries. They are consumed fresh or dried, in cocktails (in ice cubes), canned in sugar, preserved in distillates etc. Many natural antioxidants such as phenolic acids, flavonoids, anthocyanins and other phenolic compounds are present in flowers. The high antioxidant activity of flowers may be attributed to the level of flavonoid [10,15]. In the present study, we have investigated the antioxidant and antibacterial efficacy of solvent extracts of flowers of *Calycopteris floribunda* (Roxb.) Poiret, *Kydia calycina* Roxb and *Humboldtia brunonis* Wall.

MATERIALS AND METHODS

Collection and identification of plant materials

The plants employed in the present study were authenticated by Dr. Vinayaka K.S, Dept. of Botany, Indira Gandhi Government College, Sagara, Karnataka. Table 1 presents the place of collection, voucher number and reported biological activities of plants selected. The voucher specimens were deposited in the department herbaria, Dept. of Microbiology, SRNMN College of Applied Sciences, Shimoga, Karnataka.

Table 1: Plants selected for the study

Plant name	Place of collection	Voucher number	Reported biological activities
K. calicyna (Malvaceae)	Hanagerekatte, Shivamogga (D)	SRNMN/PK/Kc-006	Antifungal [16]; analgesic and anti-inflammatory [17]; hepatoprotective [18]
H. brunonis (Caesalpiniaceae)	Hulikal, Hosanagara (T), Shivamogga (D)	SRNMN/PK/Hb-020	Antimicrobial [19]; carioprotective, nephroprotective, hepatoprotective and antioxidant [20]
C. floribunda (Combretaceae)	Hosur, Sagara (T), Shivamogga (D)	SRNMN/PK/Cf-035	Cytotoxic [21]; antioxidant and β-glucuronidase inhibitory [22]; antimicrobial [23,24]; hypoglycaemic [25]; hepatoprotective [26,27]

Extraction

About 25g of each of the powdered flowers were extracted successively in Soxhlet apparatus using solvents *viz.*, petroleum ether (PE), chloroform (Chl) and methanol (Met) based on increasing polarity. The solvent extracts were filtered through Whatmann no. 1, concentrated in vacuum under reduced pressure and dried in the desiccator [28].

Antibacterial activity of solvent extracts

Antibacterial activity of solvent extracts was tested against two Gram positive bacteria *viz.*, *Staphylococcus aureus* and *Bacillus cereus* and two Gram negative bacteria *viz.*, *Pseudomonas aeruginosa* and *Escherichia coli*. The bacteria were maintained on sterile Nutrient agar (HiMedia, Mumbai) slants in refrigerator. The test bacteria were inoculated into sterile Nutrient broth (HiMedia, Mumbai) tubes and incubated overnight at 37°C and the broth cultures were screened for their susceptibility to flower extracts by Agar well diffusion assay. The test bacteria were aseptically swabbed on Nutrient agar plates using sterile cotton swabs. With the help of a sterile cork borer, wells of 6mm diameter were punched in the inoculated plates and 100µl of solvent extracts (50mg/ml of 25% DMSO), standard (Streptomycin, 1mg/ml) and DMSO (25%) were transferred into respectively labelled wells. The plates were incubated at 37°C aerobically for 24 hours and the zone of inhibition formed around the wells was measured using a ruler [29]. The experiment was repeated two times and the average reading was noted.

Antioxidant activity of solvent extracts

DPPH free radical scavenging assay

The radical scavenging ability of solvent extracts was tested on the basis of the radical scavenging effect on the DPPH free radical. 1 ml of different concentrations of flower extracts was mixed with 3 ml of DPPH solution (0.004% in methanol) in separate tubes. The tubes were incubated at room temperature in dark for 30 minutes and the optical density was measured at 517 nm using UV-Vis spectrophotometer. The absorbance of the DPPH control was also noted. Ascorbic acid was used as reference standard. The scavenging activity of the extracts was calculated using the formula:

Scavenging activity (%) = [(Ao – Ae) / Ao] x 100, where Ao is absorbance of DPPH control and Ae is absorbance of DPPH and extract/standard combination [2].

Ferric reducing assay

In this assay, various concentrations of flower extracts (5-100µg/ml) in 1 ml of methanol were mixed in separate tubes with 2.5 mL of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide (1%). The tubes were placed in water bath for 20 minutes at 50°C, cooled rapidly and mixed with 2.5 mL of trichloroacetic acid (10%) and 0.5 ml of Ferric chloride (0.1%). The amount of iron (II)-ferricyanide complex formed was determined by measuring the formation of Perl's Prussian blue at 700 nm after 10 minutes. The increase in absorbance of the reaction mixtures indicates increased reducing power. Ascorbic acid was used as standard [30].

Total Phenolic Content (TPC) of solvent extracts

The TPC of solvent extracts was determined by Folin-Ciocalteau reagent (FCR) method. A dilute concentration of extract (0.5 ml) was mixed with 0.5 ml of FC reagent (1:1) and 2 ml of sodium carbonate (7%). The reaction mixtures were allowed to stand for 30 minutes and the optical density was measured colorimetrically at 765nm. A standard curve was plotted using different concentrations of Gallic acid (standard, 0-1000 µg/ml) and the TPC of extracts was expressed as µg Gallic acid equivalents (GAE) from the graph [30].

Total Flavonoid Content (TFC) of solvent extracts

The flavonoid content of solvent extracts was estimated by Aluminium chloride colorimetric method [31]. A dilute concentration of extract (0.5ml) was mixed with 0.5ml of methanol, 4ml of water, 0.3ml of $NaNO_2$ (5%) and incubated for 5 minutes at room temperature. After incubation, 0.3ml of $AlCl_3$ (10%) was added and again incubated at room temperature for 6 minutes. 2ml of 1M NaOH and 2.4ml of distilled water were added and the absorbance was measured against blank (without extract) at 510nm using UV-Vis spectrophotometer. A calibration curve was constructed using different concentrations of Catechin (standard, 0-120 µg/ml) and the TFC of extracts was expressed as µg Catechin equivalents (CE) from the graph.

RESULTS

The color, yield, TPC and TFC of flower extracts is shown in Table 1. Sequential extraction of powdered flowers resulted in maximum yield of extracts in case of methanol solvent followed by chloroform and petroleum ether. Higher extract yields were obtained for *H. brunonis* (Hb) flowers followed by *C. floribunda* (Cf) and *K. calycina* (Kc) flowers. The amount of total phenolics present in solvent extracts of flowers was estimated by FCR method. Methanol extracts of all flowers contained higher phenolic content than other solvent extracts. Methanol extract of *H. brunonis* flower contained high phenolic content than other flower extracts. Total flavonoid content, as estimated by aluminium chloride colorimetric estimation method was also higher in methanol extract of all the flowers. Here also, *H. brunonis* contained high flavonoid content than extracts of other flowers.

Table 1: Color, Yield, TPC and TFC of solvent extracts of flowers

Extract	Color of extract	Yield of extract (%)	TPC (µg GAE/mg)	TFC (µg CE/mg)
Cf-PE	Golden yellow	0.70	25.68	14.50
Cf-Chl	Dark green	1.30	55.85	16.50
Cf-Met	Brownish orange	6.70	280.31	48.50
Kc-PE	Golden yellow	0.65	20.14	8.80
Kc-Chl	Dark green	0.70	25.68	8.80
Kc-Met	Brownish green	2.15	106.76	16.20
Hb-PE	Yellow	1.65	25.35	6.25
Hb-Chl	Green	1.85	30.61	8.75
Hb-Met	Wine red	12.00	285.33	50.75

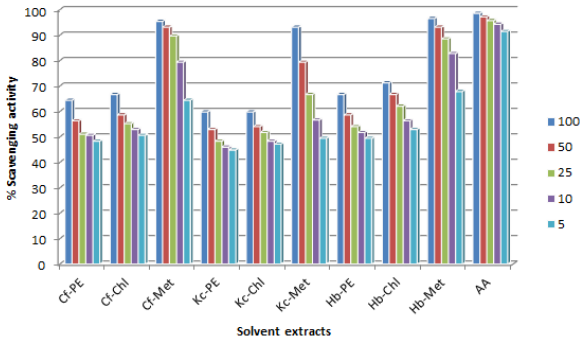
The result of antibacterial activity of solvent extracts of flowers is shown in Table 2. The results were recorded as presence or absence of zones of inhibition around the well. The inhibitory zone around the well indicated the absence of bacterial growth and it was reported as positive and absence of zone as negative. The solvent extracts of all three flowers showed inhibition of bacteria tested. Standard antibiotic Streptomycin exhibited marked inhibitory activity than solvent extracts of flowers. Among flowers, highest inhibition of bacteria was observed in case of methanol extract *C. floribunda* followed by *H. brunonis* and *K. calycina*. No inhibition of test bacteria was recorded in case of 25% DMSO (not shown in the table).

Table 2: Antibacterial activity of solvent extracts of flowers

Treatment	Zone of inhibition in cm			
	S. aureus	*B. cereus*	*P. aeruginosa*	*E. coli*
Cf-PE	2.1	2.0	2.1	2.1
Cf-Chl	2.4	1.9	2.3	2.2
Cf-Met	2.2	1.8	2.4	2.1
Kc-PE	1.4	1.2	1.4	1.2
Kc-Chl	1.7	1.3	1.7	1.5
Kc-Met	1.5	1.2	1.7	1.5
Hb-PE	1.2	1.4	1.2	1.2
Hb-Chl	1.4	1.5	1.3	1.3
Hb-Met	1.8	1.6	2.1	1.9
Str	3.8	3.6	3.2	2.9

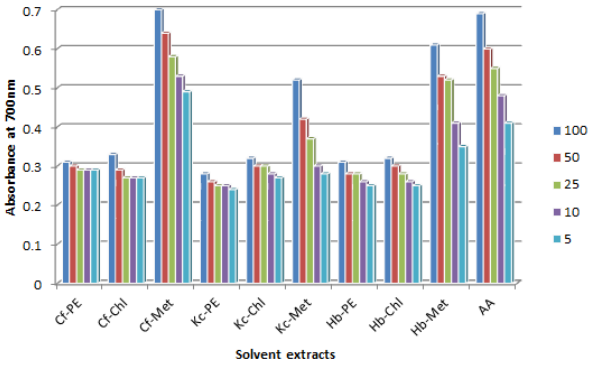
Radical scavenging activity of various concentrations of flower extracts was estimated by DPPH free radical scavenging assay. The extracts have shown dose dependent scavenging of free radicals. Among flowers, scavenging activity was highest in case of *H. brunonis* followed by *C. floribunda* and *K. calycina*. Methanol extracts of all the flowers showed higher scavenging efficacy followed by chloroform and pet ether extracts. At concentrations 100µg/ml, methanol extracts of all the flowers exhibited >90% scavenging of free radicals. Ascorbic acid scavenged radicals more efficiently than all flower extracts (Figure 1).

Figure 1: DPPH radical scavenging activity of solvent extracts of flowers



The reductive potential of flower extracts was estimated by ferric reducing assay. The absorbance of reaction mixtures containing various concentrations of flower extracts increased with increase in their concentrations and is suggestive of reducing power. Among extracts, methanol extracts have shown more reducing potential than other extracts. The reducing powers of all extracts except methanol extract of *C. floribunda* were lesser than that of ascorbic acid (Figure 2).

Figure 2: Ferric reducing activity of of solvent extracts of flowers



DISCUSSION

A situation of 'oxidative stress' occurs when the natural defences of an individual are overwhelmed by an excessive production of free radicals, particularly reactive oxygen species (ROS) such as superoxide radical, hydroxyl radical and others. This situation results in potential damage suffered by macromolecules such as proteins, lipids and nucleic acids and is implicated in several diseases or disorders [32]. The harmful effect of these free

radicals are lowered by a team of substances termed antioxidants which may be Endogenous or may be obtained from external sources such as diet or medicinal plants [3]. Antioxidants are known to act at different levels *viz*., prevention, interception and repair. Preventive antioxidants attempt to stop the formation of ROS and include superoxide dismutase and catalase. Interception of free radicals is by scavenging that involves removal of free radicals and includes effector antioxidants like vitamin C, vitamin E, glutathione, thiols, carotenoids, flavonoids and polyphenols. At the repair level, many enzymes such as Glutathione peroxidase, DNA glycosylases, DNA endonucleases, DNA ligases, DNA polymerase I and mismatch correction enzymes are involved in the repair of cellular components such as DNA or membranes [5]. Synthetic antioxidants such as butylhydroxyanisole, butylhydroxytoluene or propyl gallate have been widely used as antioxidants but are reported to be carcinogenic and mutagenic on chronic consumption. Hence, discovery of new, safe and effective antioxidants particularly from natural sources is of considerable interest in preventive medicine [33]. Recent studies have shown that consumption of fruits, vegetables, nuts, seeds, whole grains can reduce the risk of chronic diseases produced due to oxidative stress. This protective effect of plants may be attributed to the presence of natural antioxidant compounds [34,35].

Polyphenols, including flavonoids, forms a large group of naturally occurring components of the plant kingdom and are present in every part of the plants. These compounds are of considerable interest in various fields such as food, pharmacy and medicine because of wide range of biological activities including antioxidant activity. The antioxidant efficacy of phenolic compounds is chiefly due to their redox potential. These compounds are known to act as reducing agents (free radical terminators), hydrogen donors, metal chelators and singlet oxygen quenchers [9,36]. Since it has been shown that phenolic compounds of plant kingdom are one of the most effective antioxidative constituents, it is important to estimate the phenolic contents of extracts in order to assess their contribution to antioxidant activity [35]. In the present study, we have estimated total phenolic content of flower extracts by Folin-Ciocalteu reagent (FCR) method and expressed the result as mg GAE/g of dry extract. The FCR method is the commonly used colorimetric technique for estimation of total phenolic content of variety of substances including plant extracts [7,8,30,37,38,39]**.** In our study, the methanol extracts of all flowers were shown to possess higher phenolic content followed by chloroform and petroleum ether extracts. Extracts of *H.brunonis* flower contained high phenolic content than *C. floribunda* and *K. calycina*.

Flavonoids are polyphenolic compounds and consist of flavones, flavonols, flavanols, flavanone and flavanonols. These compounds represent the majority of plant secondary metabolites and have shown to possess remarkable health promotory effects such as anti-inflammatory, antioxidant, antimicrobial, anticancer and others [40]. Aluminium chloride colorimetric estimation is commonly used to quantify flavonoid content of plant extracts [41,42]. Total flavonoid contents can be determined by reaction with sodium nitrite, followed by the development of coloured flavonoid-aluminium complex formation using aluminium chloride in alkaline condition which can be monitored spectrophotometrically at maximum wavelength of 510 nm [42]. The flavonoid contents were higher in methanol extracts of all the flowers followed by other extracts. Extracts of *H. brunonis* contained higher flavonoid contents.

DPPH is a stable organic free radical having the absorption maximum band around 515-528nm (517nm). It accepts an electron or hydrogen atom and becomes a stable diamagnetic molecule [9]. It serves as a useful reagent and the model of scavenging of the stable DPPH radical is a widely used protocol for evaluation of free radical scavenging ability of various types of samples including plant extracts. The effect

of antioxidants on scavenging DPPH radical is due to their hydrogen donating ability [8]. In this test, the antioxidants reduce the purple colored DPPH radical to a yellow colored compound diphenylpicrylhydrazine, and the extent of reaction will depend on the hydrogen donating ability of the antioxidants [43]. In this study, the decrease in absorption of DPPH in the presence of various concentrations of extracts was measured at 517nm. It was found that the radical scavenging activities of all the extracts increased with increasing concentrations. It can be observed that the extracts at higher concentrations showed significant decrease in the absorbance of DPPH radical. Although the scavenging abilities of extracts were lesser than that of ascorbic acid, it was evident that the extracts showed hydrogen donating ability and therefore the extracts could serve as free radical scavengers, acting possibly as primary antioxidants [8]. In the present study, all extracts have shown dose dependent scavenging of DPPH radicals. Scavenging efficacy was comparatively higher in methanol extracts when compared to other solvent extracts. The higher scavenging activity of methanol extracts could be due to the presence of high phenolic and flavonoid contents.

In order to measure the reducing power of the extracts, we investigated the Fe^{+3}/Fe^{+2}transformation in the presence of extracts. In this assay, the presence of reductants (antioxidants) in the samples would result in the reduction of Fe^{+3} to Fe^{+2} by donating an electron. The amount of Fe^{+2}complex can be monitored by measuring the formation of Perl's Prussian blue at 700nm. Increasing absorbance at 700nm indicates an increase in reductive ability [8]. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [44]. In this study, it was found that the reducing powers of all the extracts also increased with the increase of their concentrations. It is evident that the extracts possess reductive potential and could serve as electron donors, terminating the radical chain reactions [8]. In this study, methanol extracts of all flowers exhibited stronger reducing activity than other solvent extracts. The higher reducing potential of methanol extracts could be due to high phenolic and flavonoid contents present.

Plants have been considered as an excellent source of natural compounds having diverse biological activities such as antimicrobial, antioxidant, anticancer etc. According to WHO, >80% of world's population relies on traditional medicine for their primary healthcare needs. The use of herbal medicines in various parts of the world represents a long history of human interactions with nature [45]. Plants have been used to cure several dreadful diseases by several tribal communities all over the world. Most of the modern medicines have been derived from plants or the basic molecular and active structures are based on natural products. Interest in plants with antimicrobial properties has revived as a result of problems associated with the use of antibiotics such as high cost, resistance development in pathogens and serious side effects [46,47]. As a result, a plenty of investigations have been undertaken and the studies have shown the potential of several plants to inhibit harmful microorganisms including antibiotic resistant microorganisms [45,48-52]. Medicinal plants contain components that have therapeutic significance and possess antimicrobial properties. Most important of these bioactive components are alkaloids, flavonoids, tannins, saponins and other phenolic compounds [12,45,50,53]. Phytomedicines have no apparent side effects that are associated with modern drugs. A marked antibacterial property of flower extracts was observed in this study. The inhibitory potency of solvent extracts of flowers might be attributed to the presence of phenolic compounds and flavonoids.

CONCLUSION

Drugs from plants have a long history in both traditional and modern societies as herbal remedies or crude drugs and as purified compounds. The

active principles of plants are also used as starting materials for further modifications. The drug discovery from plants still provides important new drug leads and many of which have potential clinical uses [54]. The present study revealed the antioxidant and antibacterial efficacy of solvent extracts of selected flowers. The observed activities of flowers might be attributed to the presence of secondary metabolites such as flavonoids and phenolic compounds. The flowers can be used to prevent oxidative damage caused by free radicals and to treat infections caused by pathogenic bacteria. Further studies on separation of active principles from flower extracts and their biological activity determinations are under progress.

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