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RESEARCH ARTICLE

Antimicrobial and antioxidant activities of *Kingiodendron pinnatum* (DC.) Harms and *Humboldtia brunonis* Wallich: endemic plants of the Western Ghats of India

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Abstract: The leaf and stem extracts of two endemic plants, *Kingiodendron pinnatum* (DC.) Harms and *Humboldtia brunonis* Wallich of the family Fabaceae were subjected to phytochemical analysis, antimicrobial and antioxidant assay. Phytochemicals such as phenols, flavonoids, tannins, glycosides and terpenes were present in both plants. The antibacterial and antifungal activity assays were carried out using the disc diffusion method. Antioxidant activity was carried out using DPPH radical scavenging and reducing power assays with ascorbic acid as the standard. The extracts inhibited six bacterial strains and one fungal strain. Among the solvent extracts, the methanol extract was the most effective against the tested microorganisms and it also exhibited the highest antioxidant activity. Total phenols and flavonoids were determined using the Folin-Ciocalteau and aluminium chloride methods and correlated with the antioxidant activity.

Keywords: Antimicrobial, antioxidant activity, *Kingiodendron pinnatum* (DC.), *Humboldtia brunonis* Wallich.

INTRODUCTION

Medicinal plants play an important role in promoting good health, especially in the developing countries (Oudhia & Tripathi, 1999). The medicinal value of plants are attributed to the chemically active substances that produce a definite physiological action on the human body. Several biologically active substances found in plants, including phenolic compounds (flavonoids, phenolic acids), sugars, vitamins, saponins, ethereal oils, polyunsaturated fatty acids, phospholipids, enzymes, amino acids etc., are known to possess antioxidant properties. Saponins possess specific chemical, physical and biological properties that make them useful as medicines. Phenols and flavonoids are potent antioxidants or free radical scavengers, which prevents oxidative cell damage and have strong anti-cancer activity (Okwu & Josiah, 2006). Hence, the development and utilization of more effective antioxidants of natural origin is desired (Arunachalam, 2011). Many plants have been used for their antimicrobial activity and the increasing microbial resistance to antibiotics is prompting a resurgence in the research into antimicrobial activity of plant derivatives against resistant strains (Alviano & Alviano, 2009).

Fabaceae, the third largest family of angiosperms with approximately 730 genera and over 19400 species worldwide includes the plants commonly known as legumes (Wojciechowski *et al*., 2004). Several types of alkaloids, non-protein amino acids, amines, flavonoids, isoflavonoids, coumarins, phenylpropanoids, anthraquinones, di-, sesqui- and triterpenes, cyanogenic glycosides, protease inhibitors and lectins have been described from this family (Wink, 2003). Some of the endemic plants of the Western Ghats have been reported to possess antimicrobial (Hidayathulla *et al*., 2011; Shetty *et al*., 2011; Arumugasamy, 2012) and antioxidant activities (Sukesh *et al*., 2011; Nair *et al*., 2012). *Humboldtia brunonis* and *Kingiodendron pinnatum,* well known endemic plants of the Western Ghats in India belong to this family. *H. brunonis* Wallich, a dominant myrmecophyte, commonly known as 'hasige mara' is endemic to the biodiversity hotspot of the South Western Ghats of India (Pascal, 1988). In traditional medicine this plant is used to treat menstrual problems. *K. pinnatum* (DC.) Harms known as the 'Malabar mahogany', a vulnerable and endangered medicinal plant is used in curing sores of elephants. The oleo-gum-resin of this plant

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species is used in gonorrhoea and catarrhal conditions of genito-urinary and respiratory tracts (Komal *et al*., 2011).

The paper describes the extraction and evaluation of phytochemicals from *K. pinnatum* and *H. brunonis*, and the assessment of antimicrobial and antioxidant properties of the extracts.

METHODS AND MATERIALS

Collection of plant material

The fresh leaves and stems of *K. pinnatum* (DC.) Harms and *H. brunonis* Wallich were collected from the Charmady region of the Western Ghats. The leaves and stems were shade dried, powdered and stored in air-tight polythene bags until use.

Preparation of the extract

Fifty grams of dried and powdered samples of both leaf and stem were soxhlet extracted using methanol and ethyl acetate as solvents. The samples were concentrated using a rotary evaporator. An aqueous extract was obtained by boiling 50 g of the dried and powdered sample for 8 h in a water bath and the solution was filtered through six layers of muslin cloth. The supernatant was collected and evaporated to dryness. All the extracts were stored at 4 ^{o}C until use.

Phytochemical analysis

The extracts were screened for the presence of the following phytochemicals - alkaloids (Hagers test, Wagners test, Mayers test, Dragendorff's test), flavonoids (Shinodas test), steroids (Libermann- Burchard test and Salkowski test), phenols ($FeCl_3$ test), tannins (lead acetate test), saponins (foam test), glycosides (Molisch's test, sodium hydroxide test) and resins (turbidity test) (Dey & Harborne, 1987).

Determination of total phenolic content: The total phenolic content was measured by the Folin-Ciocalteau method (Taga *et al*., 1984). A known aliquot from the stock sample (10 mg/mL) was mixed with 2.0 mL of 2 % Na_2CO_3 and allowed to stand for 2 min at room temperature. Then 100 µL of 50 % Folin Ciocalteau's phenol reagent was added. After incubation for 30 min at room temperature in darkness, the absorbance was read at 725 nm using a spectrophotometer. The total phenolic contents of the samples were expressed as mg gallic acid equivalent per gram of extract (mg GAE/g).

Determination of flavonoid content: Total flavonoid content was measured by the aluminium chloride method (Zhishen *et al*., 1999). A known volume of each extract was made up to 4 mL with distilled water and 0.3 mL of $NaNO_2$ (1:20) was added. After 5 min, 0.3 mL of 10 % $AlCl_3.H_2O$ solution was added and after 6 min, 2 mL of 1 M NaOH solution was added. The total volume was made up to 10 mL using distilled water. The absorbance against the blank was determined at 510 nm. Results were expressed as mg quercetin equivalent per gram of extract (mg QE /g).

Antimicrobial activity

The microorganisms used for the microbial sensitivity assay were Gram positive *Staphylococcus aureus* (NCIM 2079), *Bacillus subtilis* (ATCC 6633) and Gram negative *Escherichia coli* (NCIM 2931), *Pseudomonas aeruginosa* (NCIM 2200), *Klebsiella pneumoniae* (NCIM 2957) and *Proteus vulgaris* (NCIM 2813) procured from the National Chemical Laboratory, Pune, India. Two of the fungal strains *Aspergillus niger* (MTCC 1344) and *Candida albicans* (MTCC 227) were obtained from IMTECH, Chandigarh, India and *Trichoderma viridae* from the Plant Pathology Laboratory, CPCRI, Kasargod, India. The bacterial strains were maintained in nutrient agar slants and the fungal strains on potato dextrose agar slants at 4 ^{0}C in the refrigerator.

Antibacterial and antifungal assays were carried out using the disc diffusion method (Vardar-Unlu *et al*., 2003). For the *in vitro* antibacterial activity, 200 µL of overnight grown culture of each bacterium was dispensed in 20 mL of sterile nutrient broth and incubated (37 ^{o}C) for 4 − 5 hrs to standardize the culture to 10^{-5} CFU/mL. From the 24 hrs old bacterial culture 0.1 mL (10^{-5} CFU /mL) was placed on Muller Hinton agar medium (Shetty *et al*., 2011) and spread throughout the plate by spread plate technique. The dried crude extract (35 mg) was dissolved in 1 mL of dimethyl sulphoxide (DMSO) and 25 µL of the respective solvent extracts were added to the sterile discs (6 mm diameter purchased from HIMEDIA Laboratories, India) individually and aseptically. The discs were then transferred to the inoculated petri plates.

The antifungal activity was assayed by fungal inoculation on to potato dextrose agar (PDA) medium containing the discs pre-impregnated with the plant extracts. Fungal inoculum was prepared by taking 5 − 8 colonies of the fresh fungal strain from the petri dish and suspending in 5 mL of sterile distilled water. Hundred microlitres of this fungal inoculum was dispensed in 20 mL of sterile PDA medium. The discs impregnated

with the crude extracts were prepared as described above for the antimicrobial assay. The cultures were incubated for 2 days in case of *Candida albicans* and 4 − 5 days for the other two fungi.

Antimicrobial activity was recorded by measuring the diameter of the zone of inhibition. Streptomycin and nystatin (HIMEDIA) were used as positive standards against the bacterial and fungal strains, respectively.

Antioxidant Assay

DPPH radical scavenging assay (Liyana *et al*., 2005): A solution of DPPH (0.135 mM) in methanol was prepared and 1 mL of this solution was mixed with 1 mL of varying concentrations of the extracts. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm using a SYSTRONICS spectrophotometer 166 with ascorbic acid as the standard. The ability to scavenge DPPH radicals was calculated as:

$$\text{\% DPPH radical scavenging activity} = \frac{(\text{Absorbance of control - Absorbance of sample}) \times 100}{(\text{Absorbance of control})}$$

The activity was expressed as 50 % inhibitory concentration (IC_{50}) based on the percentage of DPPH radicals scavenged.

Reducing power assay (Oyaizu, 1986): Hundred microlitres of the extract from the stock solution (10 mg/mL) was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and 1 % potassium ferricyanide (2.5 mL). The mixture was incubated at 50 °C for 20 min, 2.5 mL of 10 % trichloroacetic acid added and centrifuged at 3000 rpm for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and a freshly prepared $FeCl_3$ solution (0.5 mL, 0.1 %). The absorbance was measured at 700 nm. The reducing power was expressed as ascorbic acid equivalent (AAE) milligram per gram of extract. All the experiments were triplicated.

Statistical analysis

Statistical analysis was carried out using Graph Pad Prism Software and MS excel. Correlation was used where appropriate and the differences between extract activities were compared using one way ANOVA with Bonferroni test. Differences were considered statistically significant when $p < 0.05$.

RESULTS

The percentage yield of *K. pinnatum* leaf extract from methanol, water and ethyl acetate was 16.64, 7.18 and 6.57 %, respectively, and from the stem extract it was 8.45, 18.17 and 3.48 %, respectively. The highest yield of 16.64 % was observed in the methanolic leaf extract of *K. pinnatum* and the lowest (3.48 %) in the ethyl acetate extract of the stem. The percentage yield of *H. brunonis* leaf extract obtained from methanol, water and ethyl acetate was 11.06, 10.62 and 10.95 %, and from the stem extract it was 5.22, 3.29 and 3.69 %, correspondingly. Preliminary phytochemical screening revealed the presence of phenols, flavonoids, tannins and glycosides in all the extracts from both plants. Alkaloids were absent whilst saponins and resins were reported only in the leaf extracts of *K. pinnatum* (Table 1).

The highest antibacterial activity was exhibited by the methanol leaf extract and ethyl acetate leaf extract of *K. pinnatum* against *Staphylococcus aureus* (17.16 ± 0.35 and 16.86 ± 0.05 mm). The methanol leaf extract of *H. brunonis* and ethyl acetate leaf extract of *K. pinnatum*

Table 1: Results of phytochemical screening of *Kingiodendron pinnatum* and *Humboldtia brunonis*

	KLM	HLM	KSM	HSM	KLW	HLW	KSW	HSW	KLE	HLE	KSE	HSE
Phenols	+	+	+	+	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+	+	+	+	+
Tannins	+	+	+	+	+	+	+	+	+	+	+	+
Saponins	+	–	–	–	+	–	–	–	–	–	–	–
Alkaloids	–	–	–	–	–	–	–	–	–	–	–	–
Glycosides	+	+	+	+	+	+	+	+	+	+	+	+
Steroids	+	+	+	+	_	+	–	+	+	+	+	+
Resins	+	–	+	–	–	–	–	–	–	–	–	–

+ Detected; − Not detected; K = *Kingiodendron pinnatum*; H = *Humboldtia brunonis*; L = Leaf; S = Stem; M = Methanol; E = Ethyl acetate; W = Water

Table 2: Antimicrobial activity of the extracts of *Kingiodendron pinnatum* and *Humboldtia brunonis*

Extract	Zone of inhibition (diameter in mm)								
	TV	AN	CA	BS	SA	KP	EC	PA	PV
HML	—	—	26 ± 0.3 [a]	16.66 ± 0.15 [a]	14 [a]	14.33 ± 0.57 [b]	12.33 ± 0.57 [b]	11.66 ± 0.5 [ab]	11.96 ± 0.05 [b]
HWL	—	—	15 ± 0.2 [b]	13.56 ± 0.57 [bc]	12.33 ± 0.57 [b]	10.33 ± 0.57 [a]	8.33 ± 0.57 [a]	9.00 ± 0.00 [c]	8.63 ± 0.55 [c]
HEL	—	—	20 ± 0.57 [c]	15.86 ± 0.11 [a]	13.43 ± 0.47 [bc]	11.33 ± 0.57 [a]	8.56 ± 0.85 [a]	11.06 ± 0.11 [a]	10.70 ± 0.51 [b]
HMS	—	—	25 ± 0.25 [a]	14.33 ± 0.30 [b]	12.50 ± 0.43 [b]	11 [a]	—	12.53 ± 0.30 [ab]	11.13 ± 0.15 [b]
HWS	—	—	14.8 ± 0.46 [b]	12.56 ± 0.15 [c]	9.06 ± 0.11 [d]	7.03 ± 0.05 [c]	—	9.00 ± 0.00 [c]	10.20 ± 0.26 [b]
HES	—	—	20 ± 0.00 [c]	12.80 ± 0.2 [c]	11.16 ± 0.55 [e]	10.23 ± 0.40 [a]	—	11.10 ± 0.17 [a]	10.06 ± 0.11 [b]
KML	—	—	28 ± 0.3	16.30 ± 0.52 [a]	17.16 ± 0.35 [a]	15.50 ± 0.6 [a]	15.76 ± 0.87 [c]	13.33 ± 0.57 [b]	14.86 ± 0.90 [a]
KEL	—	—	20 ± 0.2 [b]	15.46 ± 0.37 [a]	16.86 ± 0.05 [a]	13.33 ± 0.57[b]	14.30 ± 0.69 [c]	11.66 ± 0.57 [a]	14.66 ± 0.41 [a]
KWL	—	—	15 [c]	14.13 ± 0.49 [b]	14.86 ± 0.11 [c]	14.33 ± 0.57 [b]	11.87 ± 0.15 [b]	10.13 ± 0.6 [ac]	10.33 ± 0.57 [b]
KMS	—	—	20 ± 0.25 [b]	13.56 ± 0.32 [bc]	12.70 ± 0.1 [b]	10.66 ± 0.57 [a]	11.56 ± 0.58 [b]	10.00 ± 0.00 [ac]	10.90 ± 0.1 [b]
KES	—	—	14.8 ± 0.46 [c]	12.66 ± 0.15 [c]	11.20 ± 0.51 [e]	7 [c]	9.63 ± 0.63 [ad]	10.00 ± 0.00 [ac]	9.06 ± 0.11 [c]
KWS	—	—	20 ± 0.00 [b]	10.23 ± 0.15	9.33 ± 0.57 [d]	10.66 ± 0.57 [a]	10.16 ± 0.47 [bd]	9.33 ± 0.57 [ac]	9.96 ± 0.05 [bc]
Strepto-mycin 10 µg/mL				22.16 ± 0.37	25.53 ± 0.32	24.1 ± 0.1	19.13 ± 0.20	20.63 ± 0.15	30.10 ± 0.1
Nystatin 10 µg/mL	16.23 ± 0.25	18.9 ± 0.32	25.23 ± 0.20 [a]						

Results with the same letter in each column are not significant

K = *Kingiodendron pinnatum*; H = *Humboldtia brunonis*; TV - *Trichoderma viridae*; AN - *Aspergillus niger*; CA - *Candida albicans*; BS - *Bacillus subtilis*; SA - *Staphylococcus aureus*; KP - *Kliebsiella pneumoniae*; EC - *Escherichia coli*; PA - *Pseudomonas aeruginosa*; PV - *Proteus vulgaris.*

L- Leaf; S – Stem; M – Methanol; E - Ethyl acetate; W - Water; — no activity

Table 3: Antioxidant activity, phenols, and flavonoids- of *Kingiodendron pinnatum* and *Humboldtia brunonis*

Extracts	Phenols mg gallic acid equivalent/g	Flavonoids mg quercetin equivalent/g	DPPH-IC_{50}	Reducing power mg ascorbic acid equivalent/g
AA			6.53 ± 0.11 [a]	
HLM	189.51 ± 0.32 [d]	60.43 ± 0.79	7.66 ± 0.11 [a]	338.69 ± 1.54
HLE	80.99 ± 0.21 [c]	34.45 ± 1.21 [ab]	14.00 ± 1 [a]	168.42 ± 2.31 [a]
HLW	40 ± 0.8 [a]	13.51 [c]	37.33 ± 4.16 [b]	140.20 ± 4.65 [b]
HSM	68.79 ± 0.85 [b]	43.20 ± 1.99 [a]	15.06 ± 0.80 [a]	160.85 ± 2.31 [a]
HSE	61.57 ± 0.18 [b]	29.68 ± 1.65 [b]	31.33 ± 3.21 [b]	144.72 ± 3.80 [b]
HSW	32.58 ± 0.08 [a]	14.01 ± 0.86 [c]	38.00 ± 3.46 [b]	138.16 ± 0.87 [b]
KLM	360.68 ± 0.15	122.46 ± 2.10	7.20 ± 0.2 [a]	453.82 ± 5.45
KLE	182.05 ± 0.36 [d]	49.03 ± 0.45 [a]	9.06 ± 0.70 [a]	305.54 ± 1.49 [a]
KLW	143.05 ± 0.07	40.55 [a]	9.06 ± 0.11 [a]	276.88 ± 2.70
KSM	207.18 ± 2.32	48.50 ± 1.37 [a]	8.26 ± 0.30 [a]	313.13 ± 5.46 [a]
KSE	95.03 ± 1.36 [c]	41.87 ± 3.00 [a]	14.33 ± 0.57 [c]	212.29 ± 0.87
KSW	63.29 ± 1.05 [b]	30.74 ± 0.46 [b]	20.26 ± 0.92 [c]	148.75 ± 3.81

Results with the same letters in each column are not significant

K = *Kingiodendron pinnatum*; H = *Humboldtia brunonis*; AA - ascorbic acid; L – Leaf; S – Stem; M – Methanol; E - Ethyl acetate; W - Water

showed the highest zone of inhibition against *B. subtilis*. Antimicrobial activities of the leaf samples were always higher than the stem samples (Table 2). Methanolic leaf and stem extracts of *H.brunonis* and methanolic leaf extract of *K. pinnatum* exhibited anticandidal activity on par with standard Nystatin but no activity was found against *Aspergillus* and *Trichoderma*.

The highest phenolic content of 360.68 mg gallic acid equivalent/g was observed in the methanolic leaf extract of *K. pinnatum* and the lowest of 63.29 mg gallic acid equivalent/g, in the water extract of stem. The methanol extract of *H. brunonis* leaf showed a phenolic content of 189.51 mg gallic acid equivalent/g and the water extract of the stem showed a lower phenolic content of 32.58 mg gallic acid equivalent/g. Flavonoid contents of 60.43 and 122.46 mg quercetin equivalent/g were observed in the methanolic leaf extracts of *H. brunonis* and *K. pinnatum*, respectively. The IC_{50} values of 7.66 and 7.2 µg/mL for DPPH scavenging activity was observed in methanolic leaf extracts of *H. brunonis* and *K. pinnatum*, respectively. A significantly higher reducing power of 453.82 mg AAE/g for the methanolic leaf extract of *K. pinnatum* followed by 338.69 mg AAE/g for the methanolic leaf extract of *H. brunonis* were observed in the present study (Table 3).

DISCUSSION

The current study revealed the presence of phenols, flavonoids, glycosides, tannins in all extracts of *H. brunonis* and *K. pinnatum* while alkaloids were not detected. In addition, the methanolic leaf extract of *H. brunonis* showed the presence of steroids, while the methanolic leaf extract of *K. pinnatum* showed the presence of saponin and resins.

Some authors have linked the presence of phytochemicals to the antimicrobial properties of plant extracts (Adekosan *et al*., 2007). Mariita *et al*. (2010) have reported a strong antimicrobial activity (zones of inhibition between 9.00 and 14.10 mm) in *Entada abysinnica* Steudel ex A. Rich (Fabaceae) against *C. albicans*, *S. typhi* and *S. aureus.* The methanolic extract was reported to have a better zone of inhibition against *C. albicans* than fluconazole, whose zone of inhibition was 13.00 mm while no appreciable activity was reported against *E. coli* and *K. pneumoniae*. In the current study, the stem extracts of *H. brunonis* showed no activity against *E. coli*. It is suggested that the lack of appreciable activity of the plant extracts against *E. coli* may be due to the development of drug resistance through extended-spectrum β-lactamase production (Heffernan *et al*., 2009). Dyamavvanahalli *et al.* (2011) reported that the ethyl acetate leaf extract of *Humboldtia* exhibited significant antibacterial activity against Gram positive and Gram negative bacteria but no activity was observed in the methanolic leaf extract. In the present study however, both extracts showed significant activity. This may be due to the varied susceptibility of different strains of bacteria as well as species differences in plants (Karou *et al*., 2006).

The high ability of phenolics to scavenge free radicals may be due to the presence of many phenolic hydroxyl groups (Sawa *et al*., 1999). In the present study, extracts with higher phenols and flavonoids showed a high antioxidant activity. Furthermore, the total phenolic content and flavonoids were significantly higher in the methanolic extracts compared to the aqueous and ethyl acetate extracts. DPPH assay reaction depends on the ability of the samples to scavenge free radicals, which is visually noticeable as the colour changes from purple to yellow due to the hydrogen donating ability. The more rapid the absorbance decrease, the more potent the primary antioxidant activity (Saumya & Mahaboob, 2011). Methanolic extract of the leaves of *K. pinnatum* has been previously reported to have DPPH radical scavenging activity where the hydrogen donors scavenge free radical DPPH at a concentration of 0.01 mg/mL (Komal *et al*., 2011). In the present study, similar results were obtained. The IC_{50} of methanolic extract had a very high free radical scavenging activity in both plants.

In *H. brunonis,* there was no significant correlation between phenols and DPPH free radical scavenging activity ($r = -0.8032$, $p = 0.0543$) while a significant positive correlation was observed between phenols and the reducing power ($r = 0.9828$, $p = 0.0004$). Flavonoids with DPPH free radical scavenging activity showed a significant negative correlation ($r = -0.9310$, $p = 0.007$) and with reducing power, a significant positive correlation ($r = 0.8363$, $p = 0.038$). Even in *K. pinnatum*, the correlation between phenols and DPPH free radical scavenging activity was not significant ($r = -0.7757$, $p = 0.06$) whereas in the reducing power assay, it showed a significant positive correlation ($r = 0.9845$, $p = 0.0004$). Flavonoids with DPPH free radical scavenging activity showed no significant correlation ($r = -0.5589$, $p = 0.249$) and with reducing power, a significant positive correlation ($r = 0.8924$, $p = 0.016$). There was a positive linear correlation between the antioxidant activity and the total phenolic content in aqueous and methanolic extracts of selected Jordanian plant species (Tawaha *et al.,* 2007). A significant negative correlation between the phenolics content and the DPPH antioxidant activity was observed in potato varieties, indicating the role of phenolics in

radical scavenging activity (Hesam *et al.,* 2012). The significant negative correlation observed between the flavonoids and the DPPH radical scavenging activity in the present study could be due to the presence of some of the active phenolic compounds in *H. brunonis* plant extract contributing towards scavenging of free radicals.

Methanolic leaf extract of both plants showed high phenolic compounds and reasonable antimicrobial and high antioxidant activities. There is scope to pursue as the antifungal activity of methanolic extract of *Kingiodendron pinnatum*, which showed better activity compared to standard Nystatin.

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