Journal of Genetic Engineering and Biotechnology 16 (2018) 621–630

HOSTED BY

Contents lists available at ScienceDirect

Journal of Genetic Engineering and Biotechnology

journal homepage: www.elsevier.com/locate/jgeb

Original Article

Phytochemical analysis, antioxidant and antimicrobial activity of wild and *in vitro* derived plants of *Ceropegia thwaitesii* Hook – An endemic species from Western Ghats, India

S. Muthukrishnan [a,b], T. Senthil Kumar [a], A. Gangaprasad [b], F. Maggi [c], M.V. Rao [a,*]

[a] Department of Botany, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India
[b] Department of Botany, University of Kerala, Thiruvananthapuram 695 581, India
[c] School of Pharmacy, University of Camerino, Via Sant'Agostino 1, I-62032 Camerino, Italy

ARTICLE INFO

Article history:
Received 24 July 2017
Received in revised form 9 June 2018
Accepted 20 June 2018
Available online 3 July 2018

Keywords:
Ceropegia thwaitesii
In vitro and *in vivo* explants
Antioxidant potential
Phytochemical analysis
HPLC

ABSTRACT

Ceropegia thwaitesii Hook (Asclepiadaceae), an endemic plant species, due to habitat destruction and over exploitation has a very restricted distribution in the Western Ghats of Tamil Nadu, India. The present wrok aimed to determine the chemical composition, the total phenolic (TPC), flavonoid (TFC) and tannin content (TEC), and to assess the antioxidant properties of various extracts of *in vivo* plants (IVP) and *in vitro* regenerated plants (IRP) of *C. thwaitesii*. Some phenolic compounds like gallic acid, cathechol, vanillin and salicylic acid were identified and quantified by HPLC. All the extracts possessed relevant radical scavenging activity on DPPH, Superoxide radical scavenging activity, and Nitric oxide radicals as well as total antioxidant ability. DPPH assay of *in vitro* methanol stems extracts and ethanol leaves extracts revealed the best antioxidant properties with important IC_{50} values of 0.248 ± 0.45 μg/mL and 0.397 ± 0.67 μg/mL, respectively, whereas *in vivo* chloroform stems extracts showed a lower antioxidant activity (IC_{50} of 10.99 ± 0.24 μg/mL). The IRP methanol extracts of stem and leaves had good inhibitory activity against all tested microorganisms in a dose-dependent manner. These results suggested that *in vitro* raised plants of *C. thwaitesii* are an excellent source of antioxidant compounds to be exploited on an industrial level as food additive.

1. Introduction

Ceropegia L. (Asclepiadaceae) is one of the largest genera of flowering families with comprises about 200 species distributed in tropical and sub-tropical regions [1]. The exciting diversity of *Ceropegia* occurs in South Africa followed by Kenya, Madagascar, and India [2]. The genus is represented by about 50 species in India of which 35 alone occur in the Western Ghats [3]. Species of the genus *Ceropegia* are used as food, medicine and ornamental purpose [4,5]. Tubers of *C. vincaefolia* used as vegetable [6], raw tubers of *C. purpurascens* and *C. stenoloba* were edible [7]. It contains starch, sugars, gum, albuminoids, carbohydrates, crude fiber, etc., which are of nutritional importance and many species have been utilized in Ayurvedic practices in India [8,9] Such as *C. pusilla* used as Nervous weakness and *C. ciliata* plant juice used to cure the fever [10]. Cerpegin, a pyridine alkaloid was isolated from its tubers and stem. It exhibited antipyretic, hepato-protective, anti-ulcer, analgesic, hypotensive and tranquilizing activities [11–13]. The consumption and importance of wild edibles are increasing in international market due to rising recognition of its significance to human healthcare. In recent decades, extensive varieties of wild edible plants with constructive health effects have been established and marketed [14]. In spite of consumption of *Ceropegia* species by local communities in Western Ghats, such information on the nutritional value and bioactive constituents of these plants is currently not available. *Ceropegia thwaitesii* Hook, an endemic plant species, has a very restricted distribution in the Western Ghats of Tamil Nadu, India. It inhabits bare slopes of Pambar shola, and is highly vulnerable due to habitat destruction and over exploitation [15]. *Ex vivo* protocol for its multiplication has been developed and reported earlier by the author [16,17].

Worldwide the collective interest of users in traditional foods has brought about a rise in demand for traditional ingredients obtained from natural resources. The conventional collecting methods used to obtain these traditional products have several

Peer review under responsibility of National Research Center, Egypt.
* Corresponding author.
E-mail addresses: filippo.maggi@unicam.it (F. Maggi), mvrao_456@yahoo.co.in (M.V. Rao).

https://doi.org/10.1016/j.jgeb.2018.06.003
1687-157X/

disadvantages; leads to decline and scarcity of plant resources, habitat destruction and low yields. Moreover, over utilization of resources leads to plant extinction in natural. At present, *in vitro* mass propagation methods are able to overcome the above-mentioned drawbacks. The mass propagation techniques provide higher selectivity and yield, shorter times without disruption of natural habitat. Recently, many workers have reported the production of secondary metabolites in plants cultured *in vitro* [18–22]. In many cases, differentiated cultures tend to accumulate secondary metabolites in quantities higher than mother plants [23,24]. Researchers have employed various approaches including different medium strength [25] as well as the use of PGRs, elicitors and different additives [21,26,27], to stimulate the production of secondary metabolites. In *Ceropegia* species the accumulation and production of secondary metabolites *in vitro* was affected by the media types and plant species used [20]. However, there has been tiny evidence regarding phytochemical and antioxidant activities of Ceropegia species, especially leaf and stem of wild plants. The aim of the study was to estimate the phenolic compounds present in *in vivo* and *in vitro* derived plants through HPLC analysis. HPLC analysis of phenolic compounds was chosen in the present study because of simple sample treatment, possibility to adjustment the polarity of mobile phase during analysis, less analysis time and high reproducibility. Furthermore, to investigate the phenolic compounds, flavonoid, tannins and antioxidant activities of *in vivo* and *in vitro* stem and leaf extracts of *C. thwaitesii.*

2. Materials and methods

2.1. Chemicals and reagent

2.1.1. For HPLC analysis

The analytical standards of gallic acid, vanillin, p-coumaric acid, salicyclic acid and cathechol were purchased from Sigma-Aldrich (Bangalore, India). HPLC-grade methanol and water were purchased from Fisher Scientific (Mumbai, India). Before HPLC analysis, all solvents and solutions were filtered through 0.22 μm filter (Rankem, Faridabad, India).

2.1.2. For biological studies

Butylated Hydroxytoluene (BHT), Sodium carbonate, Folin-Ciocalteu reagent used for determination of phenolic content, rutin, catechin, ascorbic acid, nitro prusside, greiss reagent used for Nitric oxide radical scavenging assay were purchased from Sigma-Aldrich (Bangalore, India). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) used for the DPPH assay, aluminum chloride, potassium acetate, Con. HCL, Nitro blue tetrazolium (NBT), Nicotinamide adenine dinucleotide (NADH), Phenazine metho sulphate (PMS), sulfuric acid, sodium phosphate and ammonium molybdate were purchased from Hi-Media Laboratories (Mumbai, India).

2.1.3. Plant material and preparation of sample

Fresh plant parts (stem and leaves) of mother/*in vivo* (IVP) as well as *in vitro* raised plants (IRP) of *C. thwaitesii* were thoroughly washed under running tap water and blotted with tissue paper. For using *in vitro* plant material, the plant culture has been adapted as described earlier [16]. One gram leaves and stems of *in vitro* and *in vivo* fresh material was homogenized in 25 mL of respective organic solvent (methanol, ethanol, petroleum ether, acetone, chloroform and water) and extractions were carried on orbital shaker (REMI, India) with constant stirring at 120 rpm for overnight. The extracts were centrifuged at 10,000 rpm for 10 min and the supernatant was filtered through Whatman filter paper. The organic and aqueous extracts were concentrated and the solvent was removed using with oven. Freshly prepared extracts were used in all the experiments.

2.2. RP-HPLC analysis of phenolic compounds

The phenolic compounds were analysed by the method [28] with slight modification. One gram of fresh samples of IVP and IRP (stem and leaves) was crushed in 25 mL of methanol and extract was centrifuged at 15,000 rpm for 10 min. The supernatant was filtered through a 0.22 μm filter (Rankem, Faridabad, India) before injection into HPLC system. HPLC analyses were performed using a Waters HPLC system (Model: quaternary gradient module 2545) equipped with a Waters 717 plus auto sampler and Waters 2998 photodiode array detector. The mobile phase was methanol: water (50:50, v/v) at a flow rate of 0.8 mL min^{-1}, the injection volume set to 20 μL and the reverse phase C18 silicon column was retained at room temperature. Individual phenolic compounds were identified at 254 nm by comparing the retention time of sample chromatographic peaks with those of authentic standards analysed under the same analytical conditions.

2.3. Total phenolic content (TPC)

The determination of total phenolic content was performed by using Folin–Ciocalteu method with slight modification [29]. A 100 μL of extract was mixed with 250 μL of Folin- Ciocalteu's reagent and kept for 5 min at 25 °C. Then 750 μL of 15% Na_2CO_3 and 3.4 mL of water were added to the reaction mixture and kept for 90 min at room temperature and the absorbance was measured at 765 nm. The same procedure was repeated for standard Gallic acid solutions and total phenolic content was calculated using a calibration curve of gallic acid equivalent (GAE) (1–10 mg/mL, $y = 0.0155x - 0.2673$, $R^2 = 0.9966$, y is the absorbance of sample, x is the solution concentration). The results were expressed as mg of gallic acid equivalents GAE/g of extract.

2.4. Total flavonoid content (TFC)

Total flavonoid content was determined by colorimetric method with slight modification [30]. An extract of 500 μL, 100 μL of aluminum chloride (10%), 100 μL of potassium acetate (1 M) and 2.8 mL of distilled water were mixed for 5 min by vortexing. The reaction mixture was kept at room temperature for 30 min and the absorbance was measured at 415 nm against blank. The same procedure was repeated for standard Rutin solutions and total flavonoid content was calculated using a calibration curve of rutin equivalent (RE) (1–10 mg/mL, $y = 0.0053x - 0.3942$, $R^2 = 0.9969$, y is the absorbance of sample, x is the solution concentration). The results were expressed as mg of rutin equivalents RE/g of extract.

2.5. Total condensed tannins (TCT)

Condensed tannins were determined according to the method of [31]. To 50 μL of diluted sample, 3 mL vanillin (4%) solution and 1.5 mL of Con HCl were added; the reaction mixture was allowed to stand for 15 min at room temperature, and absorption was measured at 500 nm. For standard catechin solutions also the same procedure was followed and using a calibration curve of catechin equivalent, the total tannin content was calculated (CE) (1–10 mg/mL, $y = 0.0199x - 0.1873$, $R^2 = 0.9979$, y is the absorbance of sample, x is the solution concentration). The results were expressed as mg of catechin equivalents CE/g of extract.

2.6. Determination of antioxidant activities

2.6.1. DPPH free radical-scavenging assay

The DPPH radical scavenging activity of different extracts from IVP and IRP stems and leaves of *C. thwaitesii* was calculated

according to the method described by [32]. One ml of freshly prepared methanol solution of DPPH (1mM) was mixed with different solvent extracts at various concentrations (50–250 μg/mL). The reaction solution was shaken and incubated at 37 °C for 30 min in a dark place and absorbance was recorded at 517 nm. In every experiment, methanol was considered as blank while the ascorbic acid as control was also run simultaneously. The antioxidant BHT was used as a positive control. The DPPH activity of plant extracts was expressed as IC_{50}, the concentration of extract (μg/mL) required to scavenge 50% of DPPH radicals. IC_{50} values were estimated by a linear regression analysis. This IC_{50} values were used for remaining antioxidant assays for access the percentage of inhibition. The ability to scavenge DPPH radical was calculated by the following equation

$$\frac{Ac - As}{Ac} \times 100 \quad (1)$$

where Ac = absorbance of the blank sample, and As = absorbance of the plant extract.

2.6.2. Superoxide radical scavenging activity

The effect of solvent extracts from IVP and IRP stems and leaves of *C. thwaitesii* of superoxide radicals was determined by using the standard method [33] with some modification. The reaction mixture contained 1 mL of Nitro blue tetrazolium (NBT) solution (312 μM prepared in phosphate buffer, pH 7.4), 1 mL of Nicotinamide adenine dinucleotide (NADH) solution (936 μM prepared in phosphate buffer, pH 7.4) and 100 μL of different solvent extracts. Finally reaction was accelerated by adding 100 μL of Phenazine metho sulphate (PMS) solution (120 μM prepared in phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 °C for 5 min and absorbance was measured at 560 nm against ascorbic acid as control. The abilities to scavenge the superoxide radical were calculated using the following equation. Superoxide radical scavenging activity was calculated using the equation (Eq. (1)).

2.6.3. Nitric oxide radical scavenging assay

Nitric oxide was generated from sodium nitro-prusside and measured by the Greiss reaction. This assay was done by the method of [34]. 320 μL of extract, 360 μL of sodium nitroprusside, 216 μL of Greiss reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% napthyl ethylene diamine dihydro chloride) were mixed and incubated at 25 °C for 1 h. Finally 2 mL of water was added and absorbance was measured at 546 nm. Nitric oxide radical scavenging activities were measured using the formula (Eq. (1)).

2.6.4. Total antioxidant capacity (TAC)

The total antioxidant activity of the extract was assessed by the phosphomolybdenum method [35]. 0.3 mL of extract was mixed with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The container containing the reaction mixture were incubated at 95 °C for 90 min. Then the absorbance of the solution was measured at 695 nm. Methanol was used as blank. The activity is expressed as the number of gram equivalents of ascorbic acid. Total antioxidant activity (%) was calculated using the equation (Eq. (1)).

2.7. Antimicrobial activities

2.7.1. Microorganisms

The activity of stems and leaves extracts of IVP and IRP of *C. thwaitesii* were screened against *Escherichia coli, Vibrio cholerae, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhi, Shigella flexneri, Bacillus subtilis* and *Micrococcus luteus.*

2.7.2. Preparation of test discs

The extracts were prepared on the concentrations of 1 mg/ml. Sterile discs (6 mm in diameter) were imbued with different concentration of extracts (50, 100, 200 and 300 μg/ml respectively). The streptomycin disc was used as the positive reference for all bacterial strains.

2.7.3. Disc diffusion assay

Antibacterial activity of IVP and IRP of stems and leaves of different solvent extract was determined using disc diffusion method [36]. The overnight inoculated bacterial cultures were spread over the freshly prepared Muller-Hinton agar plates. The 6 mm sterile discs (Himedia) was kept on at centre of plate and different

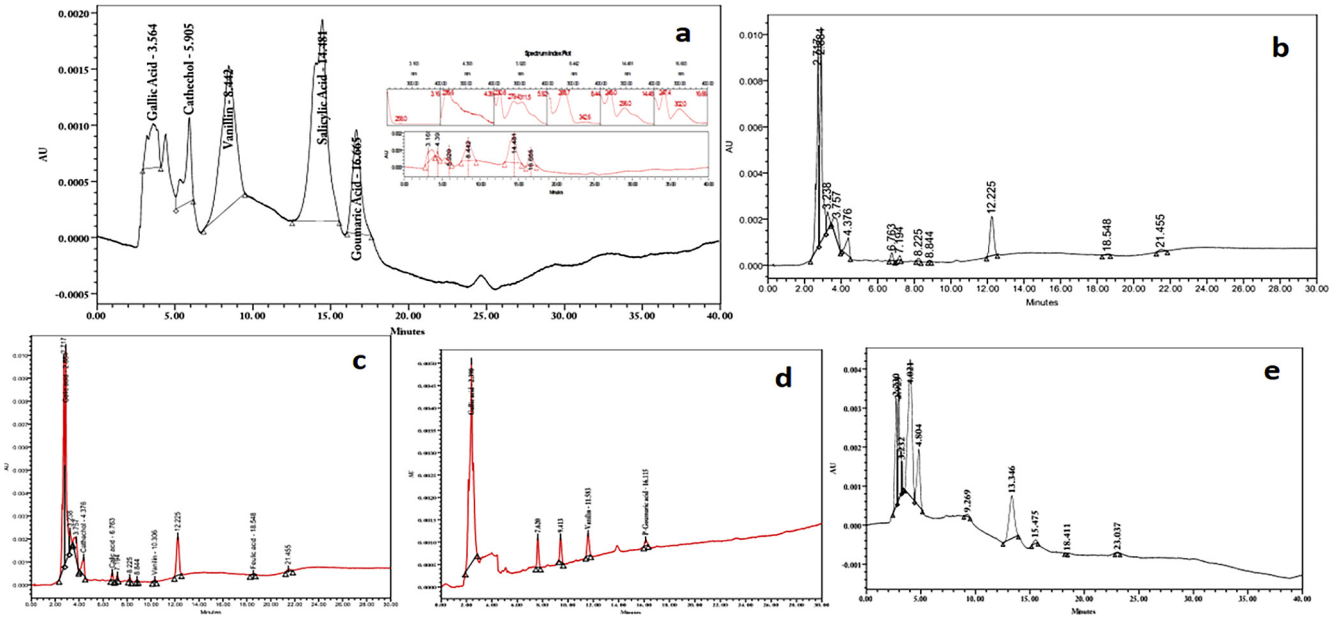

Fig. 1. RP-HPLC chromatograms of leaf and stem extracts of IVP and IRP of *C. thwaitesii*. a – Standard phenolic compounds at 1000 μg conc., (insert shows spectrum plot of individual standard); b – *in vitro*; stem; c – *in vitro* leaf; d – *in vivo* stem; e – *in vivo* leaf.

concentration of both explant solvents (50, 100, 200 and 300 μg/ml) was poured on disc. The streptomycin disc (reference disc) also kept on the plate incubated at 37 °C for 24 h. After incubation the zone of inhibition was measured.

2.8. Statistical analysis

Measurements of biochemical parameters were taken on Biotek-Synergy HT Multi-Mode Micro plate Reader with Gen5 software (Winooski-USA). The statistical analysis was carried out using the GraphPadprism5 software (GraphPad Software, Inc., USA). Each experiment was replicated three times.

3. Results and discussion

3.1. HPLC analysis of phenolic compounds

The extracts obtained from IVP and IRP of *C. thwaitesii* were subjected to HPLC analysis to evaluate its phenolic composition. Total phenolic content was highest in methanol extract, hence considered for further analysis. The major peaks were identified by comparison with authentic standards. The analysis of stems and leaves of IRP showed the presence of major phenolic compounds viz. gallic acid, vanillin, p-coumaric acid and salicylic acid (Fig. 1). The quantified content of phenolics detected in the samples is shown in Fig. 2. The compounds identified in IVP, leaves were quite similar to each other, and however the amount was significantly affected in *in vitro* regeneration. The salicylic acid had the highest content followed by Gallic acid, cathechol and vanillin in all stem and leaf of IVP and IRP sources. The highest amount of Salicylic acid (4.485 μg/mL) was present in the IRP-derived stems, followed by IVP stems and leaves (0.819 μg/mL) respectively. Salicylic acid is an endogenous signal mediating polyphenol and act against pathogens in plant systematic defense responses [37]. The presence of alkaloid and phenolic compounds in plants could be responsible for antioxidant activities [38]. Moreover, about 5 times higher gallic acid content was detected in stems as compared to the wild explants of stems and leaves. Out

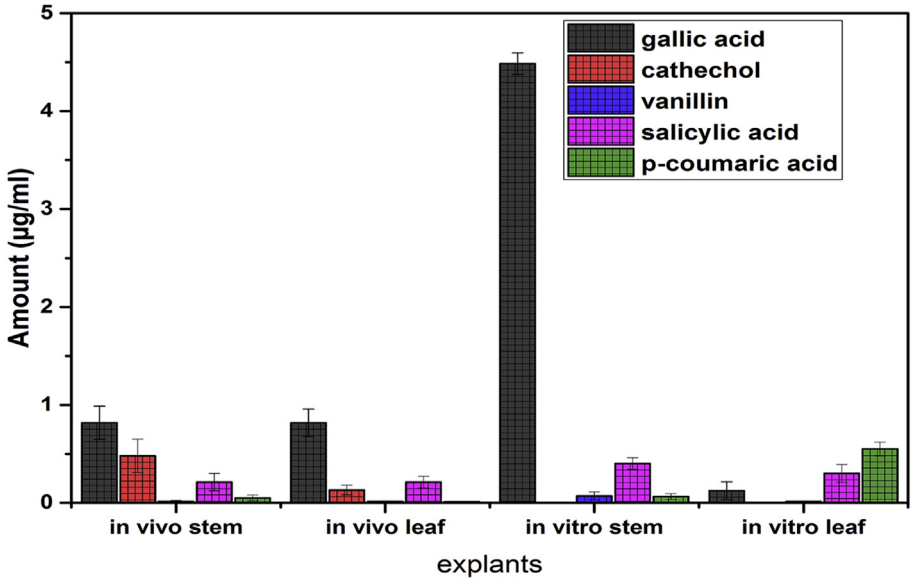

Fig. 2. The quantified content of phenolics detected in the *in vivo* and *in vitro* samples.

Table 1
Total phenolic, flavonoid and Tannin contents in Stem and leaf extracts of *C. thwaitesii*.

S. No	Plant part	Solvents	Total phenolic content (mg GAE/g FW)[a]		Total flavonoids content (mg RE/g FW)[b]		Total tannins content (mg CE/g FW)[c]	
			In vivo[*]	*In vitro*[#]	*In vivo*[*]	*In vitro*[#]	*In vivo*[*]	*In vitro*[#]
1	Stem	Ethanol	9.06 ± 1.2^{i}	11.18 ± 1.1^{j}	3.02 ± 1.4^{l}	5.01 ± 1.2^{l}	5.33 ± 2.3^{b}	9.51 ± 2.2^{b}
2		Acetone	12.55 ± 1.2^{c}	15.34 ± 2.3^{d}	40.51 ± 1.3^{c}	45.08 ± 1.1^{de}	2.45 ± 2.1^{h}	4.95 ± 2.2^{gh}
3		Pet. ether	10.41 ± 0.5^{ef}	19.38 ± 1.2^{bc}	31.92 ± 1.3^{ef}	16.51 ± 1.2^{g}	**8.57 ± 0.4^{a}**	**11.95 ± 0.4^{a}**
4		Methanol	10.27 ± 0.6^{ef}	14.94 ± 0.7^{e}	58.04 ± 1.2^{b}	60.27 ± 1.1^{b}	1.71 ± 0.6^{j}	2.34 ± 0.6^{j}
5		Chloroform	13.23 ± 0.4^{b}	14.05 ± 0.7^{e}	9.31 ± 1.3^{hi}	11.21 ± 1.1^{i}	1.19 ± 0.7^{k}	1.21 ± 0.7^{k}
6		water	**16.86 ± 1.0^{a}**	7.55 ± 0.3^{k}	6.65 ± 0.6^{jk}	7.56 ± 0.5^{k}	2.2 ± 0.6^{i}	8.87 ± 0.6^{c}
7	Leaf	Ethanol	9.08 ± 1.6^{ij}	**21.66 ± 0.4^{a}**	9.41 ± 0.6^{h}	42.7 ± 0.6^{f}	5.16 ± 0.6^{c}	7.25 ± 0.6^{d}
8		Acetone	10.65 ± 1.6^{ef}	12.32 ± 0.6^{h}	32.92 ± 0.6^{e}	45.7 ± 0.5^{d}	3.38 ± 1.1^{e}	5.87 ± 1.1^{e}
9		Pet. ether	9.73 ± 1.5^{gh}	21.66 ± 1.1^{b}	**61.12 ± 1.0^{a}**	58.62 ± 1.0^{c}	4.78 ± 1.2^{d}	5.73 ± 1.2^{ef}
10		Methanol	11.21 ± 2.3^{de}	13.82 ± 1.3^{f}	35.09 ± 1.1^{d}	**68.78 ± 1.0^{a}**	3.21 ± 1.2^{ef}	4.21 ± 1.2^{i}
11		Chloroform	11.51 ± 1.1^{de}	13.32 ± 1.5^{fg}	10.41 ± 1.3^{g}	13.32 ± 1.1^{h}	3.12 ± 1.1^{fg}	5.22 ± 1.1^{fg}
12		water	9.88 ± 0.8^{gh}	12.06 ± 1.6^{hi}	6.87 ± 1.3^{j}	8.45 ± 1.1^{j}	4.38 ± 1.1^{de}	4.69 ± 0.8^{h}

Bold values indicate the higher amount of quantified phenolic, flavonoid and tannin contents in each sample and extracts.
[a] Measurements are mean ± SE of three parallel determinations and expressed as tannic acid equivalent per gram fresh weight.
[b] Measurements are mean ± SE of three parallel determinations and expressed as rutin equivalent per gram fresh weight.
[c] Measurements are mean ± SE of three parallel determinations and expressed as catechin equivalent per gram fresh weight.
[*] Mother plant.
[#] *In vitro* raised plant; GAE – gallic acid equivalent; RE – Rutin equivalent; CE – catechin equivalen.

of the five phenolic compounds analyzed, only gallic acid, vanillin, salicylic acid and p-coumaric acid were detected in the stems and leaves of *in vitro* derived plants, whereas all the phenolic compounds were detected in *in vivo* explants but the amount of phenolic content was higher in *in vitro* derived explants. The higher amount of phenolic content could be due to the accumulation of plant growth regulators. Use of different PGRs and elicitors has been reported in a number of studies for the production and enhancement of secondary metabolites, and other useful bioactive compounds, for example, phenolic in olive plants [39] and secondary metabolite production from different plants [40]. Stem explants were best for enhancing biomass as well as production of phenolic content compared to leaves explants of *in vitro* derived *C. thwaitesii*. The higher yield of these compounds might contribute to the higher antioxidant activity. Gallic acid is a naturally occurring plant phenolic compound related to the antioxidant properties and it shows solid anticancer efficacy against human prostate cancer cells [41,42].

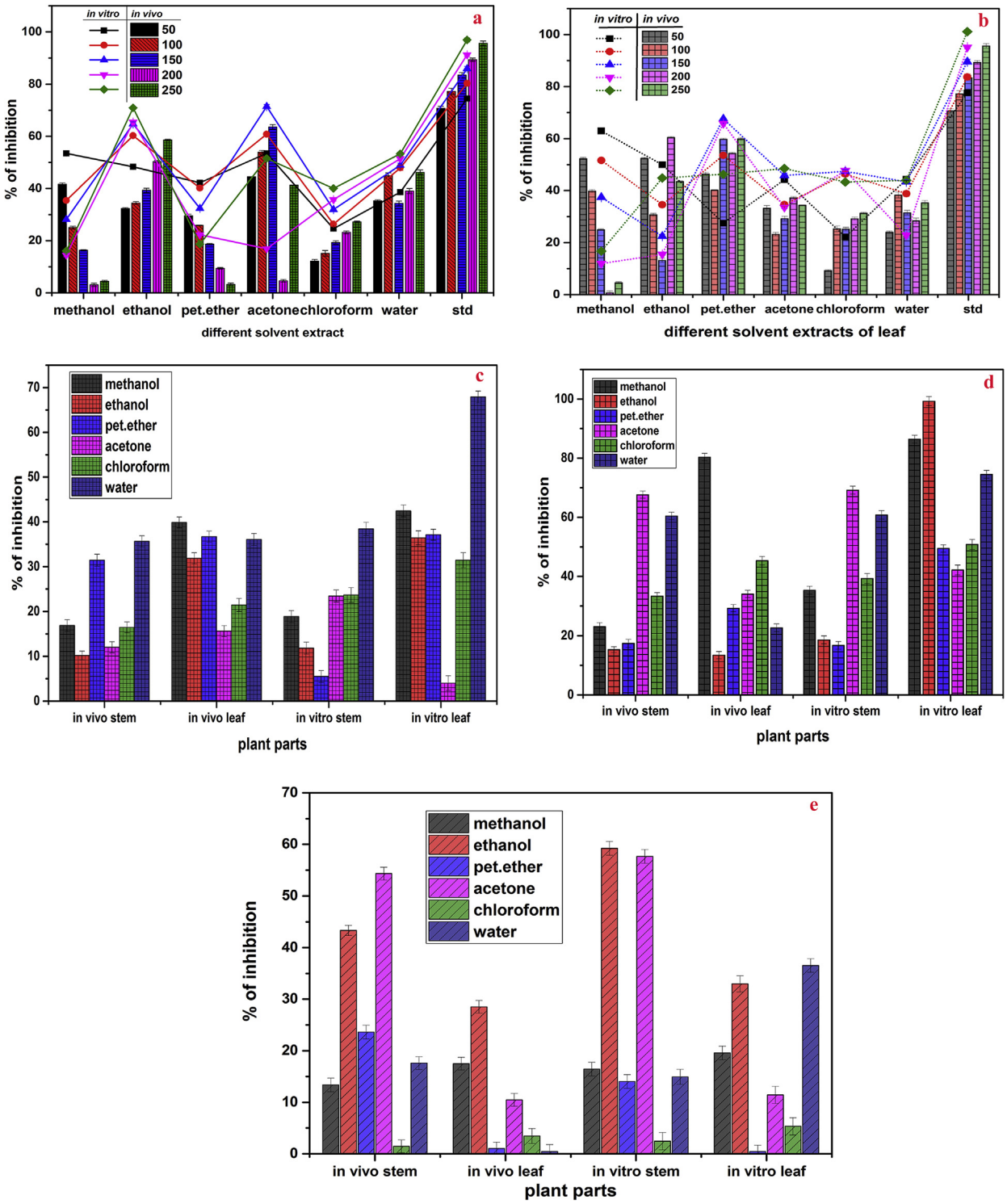

Fig. 3. Antioxidant activity of *in vitro* and *in vivo* stem and leaf extras of *Ceropegia thwaitesii*: (a) DPPH activity of stem ; (b) DPPH activity of leaf; (c) Superoxide radical scavenging activity; (d) Nitro oxide radical scavenging assay; (e) Total antioxidant capacity (TAC).

3.2. Total phenolic, flavonoid and tannin contents

The chemical composition of *C. thwaitesii* is essential in order to establish a potential relationship and understanding its role with different valuable biological activities enhancing human health benefits. Among plant secondary metabolites, phenolic compounds were shown to exert important antioxidant [43], anti-inflammatory [44], antihyperglycemic [45], immunomodulatory and anticancer [46] activity. The results showed that IVP derived plants have lower levels of phenolic substances compared to IRP (Table 1). IRP derived methanolic leaves extracts showed the highest phenolic content (21.66 ± 0.4 mg GAE/g FW) when compared to stems extracts. The phenol content decreased in the aqueous stems extracts, whereas it was higher in aqueous leaves extracts. Ethanol and acetone extracts of stems and leaves of IRP and IVP showed similar levels of phenolics. Chavan et al. [20] reported that methanol had its effect on the extraction of leaves from *in vitro* regenerated plant of *C. santapaui.* Compared to stems, leaves yielded higher values of phenolic compounds using different solvents. Likewise [47] reported high amount of phenolic compounds for leaf extracts using different solvents in three *Ceropegia* species. In Folin–Ciocalteu method phenolic undergo a redox reaction with phosphomolybdic and phosphotungstic acids present in the reagent. However, the analysis has been shown not exact to just polyphenols but to some other elements that could be oxidized by the reagent and reduced specificity of the analysis [48]. In addition, phenolic compounds, depending on the amount of phenolic groups they have, differently respond to the regent [29].

The flavonoid content was higher in the methanolic leaves extracts (60.27 ± 0.6 mg RE/g FW) followed by petroleum ether and acetone of IRP derived plants while, lower flavonoid contents were recorded in the aqueous stems and leaves extracts of IVP and IRP derived plants. The total content of flavonoid was higher in the leaves extracts of IRP than compared to stems extracts of IRP. Significantly liquefaction of flavonoids was affected by extraction of different solvents used in this experiment and these reports are in accordance with the results obtained for three *Ceropegia* species [47] and *Limonium delicatulum* [49]. Very recent reports and our own results on phenolics and flavonoids having antioxidants properties and activities. In this respect, they both may stimulate and inhibit oxidative reactions strongly. They also able to act as radical scavengers or radical-chain breakers, these capability present result demonstrated the strong antioxidant activity. Apart from phenolics and flavonoids, tannins are also widely distributed and very important plant phytochemical. Tannins show an effective antibacterial [50] and high antioxidant activity [51]. Apart from these effects, tannins also have antinutritional effect [52]. In *Ceropegia*, this is the first report related to preliminary quantification of tannin. The tannin content was higher in petroleum ether stems extracts (11.95 ± 0.1 CE/g FW) followed by methanol and aqueous extracts of IRP derived plants and leaves extracts of all the solvents from IRP (Table 1). Although tannins in plants function as the electron supplier for the antioxidative enzymes, they act as a backup defense mechanism of plants [53]. Tannins are naturally occurring phenolic compounds and have many phenolic groups which precipitate protein due to high molecular weight (M_r > 500). Tannins (polymeric polyphenolics) may be much more potent antioxidants than are simple monomeric phenolics [51].

The fluctuation of biochemical content in the different solvent extracts is based on a number of intrinsic and extrinsic factors and specific metabolic activities as well as endogenous physiological changes in the plants [54]. Similarly, the difference of phenol and flavonoid contents within the plant parts was reported in 12 plants of family Asclepiadaceae and Periplocaceae [55]. Present results indicate that, exogenous supply of different PGRs during *in vitro* regeneration, pathway of propagation was markedly influenced the *in vitro* production of phenols and flavonoids. Similarly, influence of PGRs on *in vitro* production of secondary metabolites from callus suspension culture is reported in *Gymnema sylvestre* [27,56]. The present report has shown the total phenolic, flavonoid and condensed tannin contents were higher in *in vitro* regenerated plants than mother plant or *in vivo* plants of *C. thwaitesii.* From these results, we concluded that PGRs enhance the phytochemical, these probably PGRs act as precursors to their conversion to active forms during stress or adverse environmental conditions.

3.3. Determination of antioxidant activities

3.3.1. DPPH radical scavenging activity

The method of scavenging DPPH radical is specifically used to estimate chain-breaking activity in the proliferation phase of lipid (and protein) oxidation. The antioxidant effects on DPPH scavenging was thought to be due to ability of their hydrogen donation capacity [57]. All the extracts exhibited a dose-dependent increase in DPPH scavenging activity (Fig. 3a and b). IRP extracts had a significant effect on DPPH radical scavenging activity, in particularly IRP pet. ether leaves and ethanol stems extract has 84% of activity that virtually proportionate to commercial antioxidant BHT (89%) at concentration of 250 μg/mL. Table 2 shows antioxidant activity with IC_{50} values of *in vivo* and *in vitro* of stems and leaves measured by DPPH radical-scavenging assays. Overall, *in vitro* methanol stems extract revealed the best antioxidant properties (significantly lower IC_{50} values = 0.248 ± 0.45 μg/mL) and the *in vitro* leaves ethanol extracts possess moderate radical scavenging activity (0.397 ± 0.67 μg/mL). *In vivo* chloroform stems extracts revealed a poor antioxidant activity (significantly higher IC_{50} values = 10.99 ± 0.24 μg/mL). This showed that the *in vitro* stem and leaves extract exhibit a strong scavenging activity than all the extracts of wild plants. During *in vitro* cultivation stress conditions

Table 2
The antioxidant activity with IC_{50} values of *in vivo and in vitro of stem and leaf measured by* DPPH radical-scavenging assays. Overall, *in vitro* raised plants revealed the best antioxidant properties (significant IC_{50} values = 0.248 μg/ml), and *in vivo* plants revealed the poor antioxidant property (significantly lower IC_{50} values = 10.99 μg/ml).

Solvents	Stem (μg/ml)		Leaf (μg/ml)	
	In vivo[a]	*In vitro*[b]	*In vivo*[a]	*In vitro*[b]
Methanol	0.306 ± 1.02	0.248 ± 0.45	1.084 ± 2.03	1.39 ± 0.56
Ethanol	4.023 ± 0.08	1.797 ± 0.45	0.563 ± 0.98	0.397 ± 0.67
Pet. ether	1.725 ± 0.28	0.800 ± 0.56	2.447 ± 0.78	2.196 ± 0.76
Acetone	1.494 ± 0.23	1.952 ± 0.89	6.330 ± 0.78	5.438 ± 0.68
Chloroform	10.99 ± 0.24	8.998 ± 0.78	8.123 ± 0.24	5.347 ± 0.12
Water	9.225 ± 0.34	3.456 ± 0.67	6.124 ± 0.45	3.835 ± 0.23

Measurements are mean ± SE of triplicate determinations, IC_{50} concentration required for inhibit the radical formation by 50%.
[a] Mother plant.
[b] *In vitro* raised plant.

may have stimulated polyphenol production, and PGRs treatment might have been responsible. PGRs levels in tissue culture media responsible for the formation and stimulation of some phenolic compounds [58]. Natural antioxidants play a crucial role in ameliorating the detrimental effects of oxidative stress caused by reactive oxygen species. There is a solid relationship between pathogenic agents and oxidative pressure levels; hence the usage of natural antioxidant is considered an effective therapeutic approach against several diseases [59]. The prospective use of *in vitro* technology for the synthesis of antioxidant compounds from wild medicinal plants has been generally reported in recent years [18,60]. The increase concentration of extract as well increased the scavenging

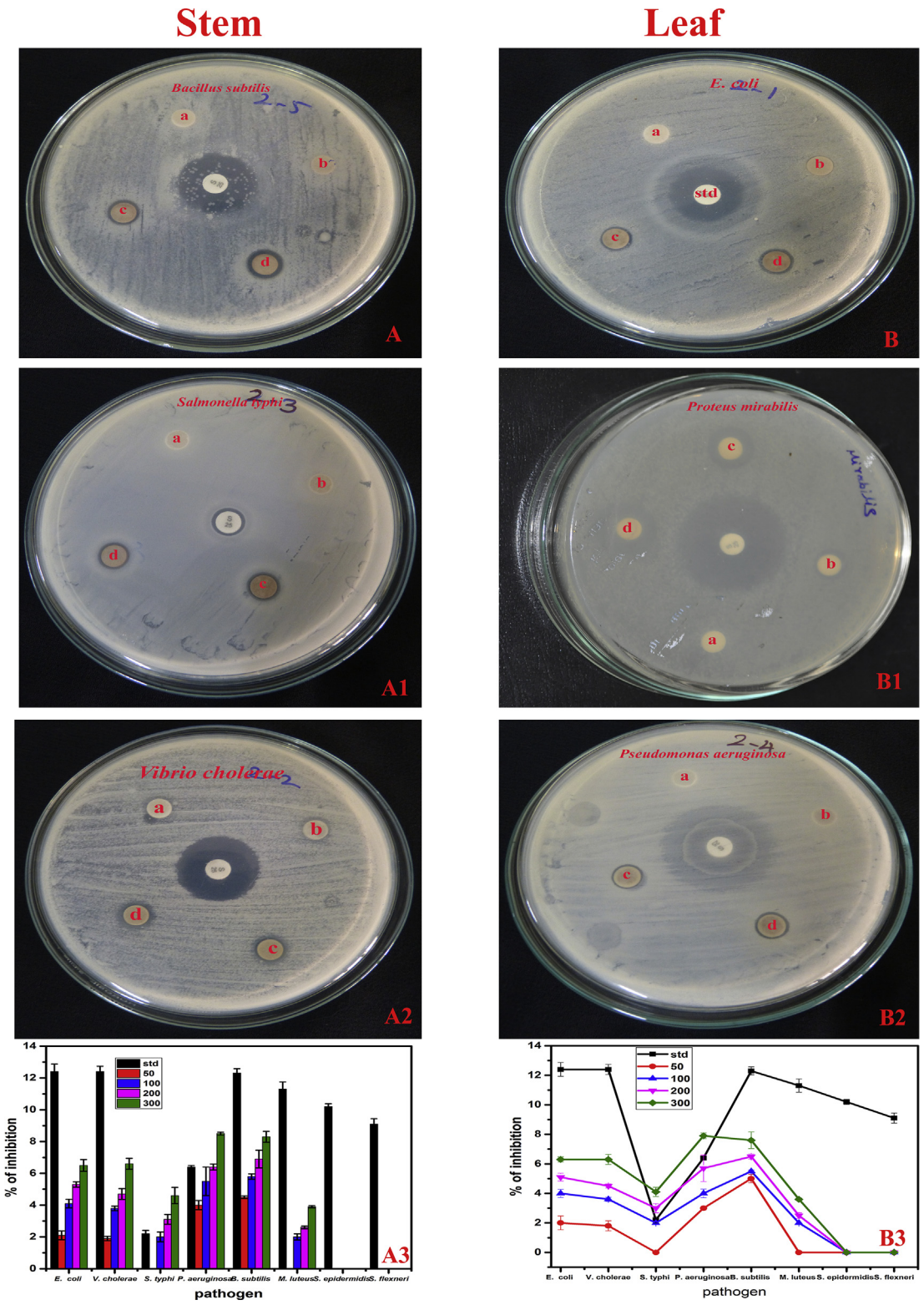

Fig. 4. Antimicrobial activity of *in vitro* raised plant (IRP) stem and leaf methanol extract against various pathogenic bacterial strains: A–A3 effects of methanol stem extract ; B–B3 effects of leaf methanol extract : (a) 50 µg/ml; (b) 100 µg/ml; (c) 200 µg/ml; (d) 300 µg/ml; Standard antibiotic (Centre).

activity due to the presence of phenols and flavonoids in extracts. The results discovered that ethanol extract of IRP stems is a strong free radical scavenger compared to other extracts. [61] reported *in vitro* regenerated plant extracts possessed the strong effects on reducing DPPH radical scavenging than *in vivo* plants.

3.3.2. Superoxide radical scavenging activity

The different extracts of IVP and IRP of stems and leaves showed potent superoxide scavenging activity in an explant and solvent dependent manner (Fig. 3c). Aqueous extract of IRP leaves had a highest activity followed by methanol, ethanol and pet. ether. Leaves and stems extracts of IRP had moderate activity compared to IVP extracts.

Aqueous extract exhibited a strong superoxide radical activity in comparison with other extracts, which could be due to a better solubility of antioxidant compounds in water. Wong et al. [62] reported that aqueous extracts of 23 from 30 medicinal plants have higher antioxidant activity than those of the other extracts.

3.3.3. Nitric oxide radical scavenging assay

Nitric oxide radical which is a produced *in vivo* by range of cell types, is a significant bioregulatory molecule with an amount of physiological role. However, under oxidative stress this RNS reacts with other reactive species to produce more toxic reactive nitrogen species (RNS) and reactive oxygen species (ROS) [63]. In the present experiment, solvent extracts of the *C. thwaitesii* stem and leaf parts were tested for its inhibitory effect on nitric oxide production. Among the oxygen radicals, nitric oxide is one of the most compassionate and persuade harsh blow to contiguous biomolecules. In this study, ethanol extracts (100 μg/mL) of IRP leaves scavenged hydroxyl radicals high while the other leaves extracts of methanol, aqueous, chloroform, pet. ether and acetone had moderate scavenging activity when compare to the IRP stems extracts. This result expressed that the different solvent extracts of leaves and stems of IRP had high scavenging activity than compare to stems and leaves of IVP (Fig. 3d).

The antioxidant principles present in the extract compete with O_2 that reacts with nitrogen to form NO; thereby inhibiting the generation of nitrites and this was indicated by less colour development with Greiss reagent compared with that of the positive control. As scavengers of nitrite radicals, leaves can protect humans from a number of diseases, as excess NO is associated with several diseases [64]. It is likely that the ethanolic extract of leaves inhibited the generation of nitric acid in the *in vitro* reaction mixture.

3.3.4. Total antioxidant content (TAC)

The TAC was also significantly different among IVP and IRP. The total antioxidant capacity of *C. thwaitesii* was much higher in stems and leaves of IRP plants (Fig. 3e) and varied according to the solvent. For example, ethanol extract of stem from IRP had the maximum total antioxidant activity, followed by the acetone extract of stem, ethanol and aqueous extracts of IRP leaves. For explants from *in vivo* the acetone extract of stem had the most activity, followed by ethanol, pet. ether and ethanol extract of leaves. Moreover the stems extract of IRP had higher total antioxidant than stems and leaves extracts of IVP.

This study exhibited the significantly high TAC in IRP leaves and stems. Using the phospho molybdenum model for measuring antioxidant activity leaf and stem extracts of IRP showed higher antioxidant activity when compared to the IVP. Likewise [18] reported total antioxidant activity of *in vitro* regenerated plants. The extracts revealed electron-donating ability and thus they may act as radical chain terminators, transforming reactive free radical species into extra stable non-reactive products [65]. The total antioxidant capacity of plant extracts may be recognized to their chemical composition and phenolic content.

3.4. Antimicrobial activity

Fig. 4 shows the antibacterial activity of extracts of IVP and IRP of *C. thwaitesii*. As the initial results showed that *C. thwaitesii* at the IRP explants had a higher phenol content and greater antioxidant activity than plants in the IVP stem and leaf. So, the antimicrobial activity was evaluated on leaves and stem extracts of IRP. Hexane, acetone and aqueous extracts had no antimicrobial activity, ethanol and acetone extracts had moderate activity against *P. aeruginosa* and *M. luteus*, while methanol extracts of stems and leaves had good inhibitory activity against all tested microorganisms. The antibacterial potential was dose-dependent against *Salmonella, Pseudomonas* and *Micrococcus*. Form these results methanol extracts were found to be the most effective, with a broad antimicrobial spectrum against both Gram positive and Gram-negative bacteria and the most susceptible bacteria being *Salmonella* and *Pseudomonas*. This finding is important, because these bacteria are resistant to a number of antibiotics and produce toxins that cause many types of enteritis and septicaemia. Phenols have previously been reported to have a wide spectrum of biological activity, including anti-thrombotic, cardioprotective, vasodilator and antimicrobial activities [66,67]. The antimicrobial activity of the extract may be attributed to the high content of flavonoids, which have been reported to be involved in inhibition of nucleic acid biosynthesis and other metabolic processes and also inhibit spore germination of plant pathogens [68,69].

4. Conclusion

The present study reports the phytochemical analysis, antioxidant activity and antimicrobial activity of IVP and IRP (stem and leaves) of *C. thwaitesii*, an endemic species from Western Ghats. Extracts obtained from IVP and IRP showed phenolic, flavonoid, tannin and antioxidant capacity with different efficiencies. The leaves and stem extract of the IRP and IVP had phenolic, flavonoid and tannin contents, antioxidant capacity and antimicrobial activity. Gallic acid, vanillin and salicylic acids were the major phenolic compounds in the extracts of IVP and IRP, but their quantities were higher in IRP extracts due to PGRs effect. The type of explant and PGRs had profound effect on the level of phytochemical at *in vitro* regenerated plants. The higher amount of phytochemicals in stems of *in vitro* propagated plants indicated that, the *in vitro* propagation offering advantages for present and future pharmaceutical industry over conventional propagation.

Acknowledgements

The authors thank Prof. N. Jayabalan, Co-ordinator, UGC-SAP, Department of Botany, Bharathidasan University, Tiruchirappalli for providing HPLC facility and Mr. G. Siva, for technical service rendered. The first author thankful to the DST-SERB New Delhi for providing financial support through National-Post Doctoral Fellowship (N-PDF) (Ref: File No. PDF/2016/002503 dt. 28/03/2017). One of the authors (M.V. Rao) is thankful to University Grants Commission, New Delhi for Emeritus Fellowship.

Conflict of interest

No conflict of interest.

References

[1] Bruyns P. Three new succulent species of Apocynaceae (Asclepiadoideae) from southern Africa. Kew Bull 2003:427–35.

[2] Murthy KSR, Kondamudi R, Karuppusamy S, Pullaiah T. Check-list and conservation strategies of the genus Ceropegia in India. Int J Biodivers Conserv 2012;4:304–15.
[3] Karthikeyan S, Sanjappa M, Moorthy S. Flowering plants of India. Dicotyledons: vol. 1. Acanthaceae-Avicenniaceae. Botanical Survey of India; 2010.
[4] Hodgkiss RJ; 2004, http://www.succulent-plant.com/ceropg.html.
[5] http://www.sagereynolds.com/cero/clist.com.
[6] Jagtap S, Deokule S, Bhosle S. Some unique ethnomedicinal uses of plants used by the Korku tribe of Amravati district of Maharashtra, India. J Ethnopharmacol 2006;107:463–9.
[7] Aiyambo D. Traditional uses of selected members of the Apocynaceae family in Namibia. Windhock: Ministry of Agriculture, Water and Forestry; 2010.
[8] Mabberley DJ. Mabberley's plant-book. Cambridge University Press; 2008.
[9] Jain SK, Defillips RA. Asclepiadaceae. In: Algonae MI, editor. Medicinal plants of India. Michigan (USA): Reference Publications Inc; 1991.
[10] Rajan S, Jayendran M, Sethuraman M. Folk herbal practices among Toda tribe of the Nilgiri Hills in Tamil Nadu, India. J Natural Remed 2005;5:52–8.
[11] Adibatti N, Thirugnanasambantham P, Kulothungan C, Viswanathan S, Kameswaran L, Balakrishna K, Sukumar E. A pyridine alkaloid from Ceropegia juncea. Phytochemistry 1991;30:2449–50.
[12] Sukumar E, Gopal R, Rao R, Viswanathan S, Thirugnanasambantham P, Vijayasekaran V. Pharmacological actions of cerpegin, a novel pyridine alkaloid from Ceropegia juncea. Fitoterapia 1995;66:403–6.
[13] Nadkarni KM. Indian materiamedica. Bombay: Popular Prakashan; 1976.
[14] Nebel S, Pieroni A, Heinrich M. Ta chòrta: wild edible greens used in the Graecanic area in Calabria, Southern Italy. Appetite 2006;47:333–42.
[15] Ahmedull M, Nayar MP. Red data book for Indian plants. Calcutta: Botanical Survey of India; 1999.
[16] Muthukrishnan S, Benjamin JF, Muthukumar M, Rao MV. In vitro propagation of Ceropegia thwaitesii Hook-an endemic species of Western Ghats of Tamil Nadu, India. Afr J Biotechnol 2012;11:12277–85.
[17] Muthukrishnan S, Kumar TS, Rao MV. An efficient in vitro regeneration and ex vitro rooting of Ceropegia thwaitesii: an endemic species from Western Ghats. Int J Pharm Sci Rev Res 2015;30:202–11.
[18] Amoo S, Aremu A, Van Staden J. In vitro plant regeneration, secondary metabolite production and antioxidant activity of micropropagated Aloe arborescens Mill. Plant Cell Tissue Organ Cult (PCTOC) 2012;111:345–58.
[19] García-Pérez E, Gutiérrez-Uribe JA, García-Lara S. Luteolin content and antioxidant activity in micropropagated plants of Poliomintha glabrescens (Gray). Plant Cell, Tissue Organ Cult (PCTOC) 2012;108:521–7.
[20] Chavan J, Gaikwad N, Umdale S, Kshirsagar P, Bhat K, Yadav S. Efficiency of direct and indirect shoot organogenesis, molecular profiling, secondary metabolite production and antioxidant activity of micropropagated Ceropegia santapaui. Plant Growth Regulat 2014;72:1–15.
[21] Coste A, Vlase L, Halmagyi A, Deliu C, Coldea G. Effects of plant growth regulators and elicitors on production of secondary metabolites in shoot cultures of Hypericum hirsutum and Hypericum maculatum. Plant Cell Tissue Organ Cult (PCTOC) 2011;106:279–88.
[22] Baskaran P, Moyo M, Van Staden J. In vitro plant regeneration, phenolic compound production and pharmacological activities of Coleonema pulchellum. S Afr J Bot 2014;90:74–9.
[23] Collin H. Secondary product formation in plant tissue cultures. Plant Growth Regulat 2001;34:119–34.
[24] Matkowski A. Plant in vitro culture for the production of antioxidants—a review. Biotechnol Adv 2008;26:548–60.
[25] Baque MA, Lee E-J, Paek K-Y. Medium salt strength induced changes in growth, physiology and secondary metabolite content in adventitious roots of Morinda citrifolia: the role of antioxidant enzymes and phenylalanine ammonia lyase. Plant Cell Rep 2010;29:685–94.
[26] Ahmed A, Rao AS, Rao MV, Taha RM. Production of Gymnemic acid depends on medium, explants, PGRs, color lights, temperature, photoperiod, and sucrose sources in batch culture of Gymnema sylvestre. Sci World J 2012;2012.
[27] Ali Ahmed AB, Rao AS, Rao MV. In vitro production of gymnemic acid from Gymnema sylvestre (Retz) R. Br. ex Roemer and Schultes through callus culture under abiotic stress conditions. In: Protocols in vitro culturzes and secondary metabolite analysis of aromatic and medicinal plants; 2009. p. 93–105.
[28] Ghatge SR. In vitro cultural studies in medicinal plants viz. Hemidesmus indicus (L.) Schult and Rubia cordifolia L. Ph.D. thesis, submitted to Shivaji University, Kolhapur; 2007.
[29] Singleton V, Orthofer R, Lamuela-Raventos R. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol. 1999;299:152–78.
[30] Woisky RG, Salatino A. Analysis of propolis: some parameters and procedures for chemical quality control. J Apicult Res 1998;37:99–105.
[31] Sun B, Ricardo-da-Silva JM, Spranger I. Critical factors of vanillin assay for catechins and proanthocyanidins. J Agric Food Chem 1998;46:4267–74.
[32] Brand-Williams W, Cuvelier M-E, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT – Food Sci Technol 1995;28:25–30.
[33] Nishikimi M, Rao NA, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys Res Commun 1972;46:849–54.
[34] Noguchi N, Nishino K, Washio E, Shi H, Chen J, Niki E. Antioxidant action of Ginkgo biloba extract. Nihon Yukagaku Kaishi 1997;46:1481–8.
[35] Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal Biochem 1999;269:337–41.
[36] Bauer A, Kirby W, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 1966;45:493.
[37] Rivas-San Vicente M, Plasencia J. Salicylic acid beyond defence: its role in plant growth and development. J Exp Bot 2011;62:3321–38.
[38] Akgul C, Saglikoglu G. Antibacterial activity of crude methanolic extract and its fractions of aerial parts of Anthemis tinctoria. Indian J Biochem Biophys 2005;42:395.
[39] Del Río J, Báidez A, Botía J, Ortuno A. Enhancement of phenolic compounds in olive plants (Olea europaea L.) and their influence on resistance against Phytophthora sp.. Food Chem 2003;83:75–8.
[40] Mulabagal V, Tsay H-S. Plant cell cultures-an alternative and efficient source for the production of biologically important secondary metabolites. Int J Appl Sci Eng 2004;2:29–48.
[41] Jafri L, Saleem S, Ullah N, Mirza B. In vitro assessment of antioxidant potential and determination of polyphenolic compounds of Hedera nepalensis K. Koch. Arab J Chem 2014.
[42] Ji B-C, Hsu W-H, Yang J-S, Hsia T-C, Lu C-C, Chiang J-H, Yang J-L, Lin C-H, Lin J-J, Suen L-JW. Gallic acid induces apoptosis via caspase-3 and mitochondrion-dependent pathways in vitro and suppresses lung xenograft tumor growth in vivo. J Agric Food Chem 2009;57:7596–604.
[43] Kähkönen MP, Hopia AI, Vuorela HJ, Rauha J-P, Pihlaja K, Kujala TS, Heinonen M. Antioxidant activity of plant extracts containing phenolic compounds. J Agric Food Chem 1999;47:3954–62.
[44] Fu R, Zhang Y-T, Guo Y-R, Huang Q-L, Peng T, Xu Y, Tang L, Chen F. Antioxidant and anti-inflammatory activities of the phenolic extracts of Sapium sebiferum (L.) Roxb. leaves. J Ethnopharmacol 2013;147:517–24.
[45] Manickam M, Ramanathan M, Farboodniay Jahromi M, Chansouria J, Ray A. Antihyperglycemic activity of phenolics from Pterocarpus marsupium. J Natural Prod 1997;60:609–10.
[46] Liu X, Zhao M, Wu K, Chai X, Yu H, Tao Z, Wang J. Immunomodulatory and anticancer activities of phenolics from emblica fruit (Phyllanthus emblica L.). Food Chem 2012;131:685–90.
[47] Chavan JJ, Gaikwad NB, Kshirsagar PR, Dixit GB. Total phenolics, flavonoids and antioxidant properties of three Ceropegia species from Western Ghats of India. S Afr J Bot 2013;88:273–7.
[48] Escarpa A, González M. Approach to the content of total extractable phenolic compounds from different food samples by comparison of chromatographic and spectrophotometric methods. Anal Chim Acta 2001;427:119–27.
[49] Medini F, Fellah H, Ksouri R, Abdelly C. Total phenolic, flavonoid and tannin contents and antioxidant and antimicrobial activities of organic extracts of shoots of the plant Limonium delicatulum. J Taibah Univ Sci 2014;8:216–24.
[50] Scalbert A. Antimicrobial properties of tannins. Phytochemistry 1991;30:3875–83.
[51] Hagerman AE, Riedl KM, Jones GA, Sovik KN, Ritchard NT, Hartzfeld PW, Riechel TL. High molecular weight plant polyphenolics (tannins) as biological antioxidants. J Agric Food Chem 1998;46:1887–92.
[52] Serrano J, Puupponen-Pimiä R, Dauer A, Aura AM, Saura-Calixto F. Tannins: current knowledge of food sources, intake, bioavailability and biological effects. Mol Nutrit Food Res 2009;53:S310–29.
[53] Sakihama Y, Cohen MF, Grace SC, Yamasaki H. Plant phenolic antioxidant and prooxidant activities: phenolics-induced oxidative damage mediated by metals in plants. Toxicology 2002;177:67–80.
[54] Fratianni F, Tucci M, De Palma M, Pepe R, Nazzaro F. Polyphenolic composition in different parts of some cultivars of globe artichoke (Cynara cardunculus L. var. scolymus (L.) Fiori). Food Chem 2007;104:1282–6.
[55] Surveswaran S, Cai Y-Z, Xing J, Corke H, Sun M. Antioxidant properties and principal phenolic phytochemicals of Indian medicinal plants from Asclepiadoideae and Periplocoideae. Natural Prod Res 2010;24:206–21.
[56] Nagella P, Murthy HN. In vitro production of gymnemic acid from cell suspension cultures of Gymnema sylvestre R. Br Eng Life Sci 2011;11:537–40.
[57] Mao L-C, Pan X, Que F, Fang X-H. Antioxidant properties of water and ethanol extracts from hot air-dried and freeze-dried daylily flowers. Eur Food Res Technol 2006;222:236–41.
[58] Barz W. Degradation of polyphenols in plants and plant cell suspension cultures. Physiol Vegetale 1977.
[59] Moyo M, Ndhlala AR, Finnie JF, Van Staden J. Phenolic composition, antioxidant and acetylcholinesterase inhibitory activities of Sclerocarya birrea and Harpephyllum caffrum (Anacardiaceae) extracts. Food Chem 2010;123:69–76.
[60] Abbasi BH, Khan MA, Mahmood T, Ahmad M, Chaudhary MF, Khan MA. Shoot regeneration and free-radical scavenging activity in Silybum marianum L. Plant Cell Tissue Organ Cult (PCTOC) 2010;101:371–6.
[61] Grzegorczyk I, Matkowski A, Wysokińska H. Antioxidant activity of extracts from in vitro cultures of Salvia officinalis L. Food Chem 2007;104:536–41.
[62] Wong C-C, Li H-B, Cheng K-W, Chen F. A systematic survey of antioxidant activity of 30 Chinese medicinal plants using the ferric reducing antioxidant power assay. Food Chem 2006;97:705–11.
[63] Dastmalchi K, Dorman HD, Oinonen PP, Darwis Y, Laakso I, Hiltunen R. Chemical composition and in vitro antioxidative activity of a lemon balm (Melissa officinalis L.) extract. LWT – Food Sci Technol 2008;41:391–400.
[64] Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993.
[65] Dorman HD, Kosar M, Kahlos K, Holm Y, Hiltunen R. Antioxidant properties and composition of aqueous extracts from Mentha species, hybrids, varieties, and cultivars. J Agric Food Chem 2003;51:4563–9.

[66] García-Lafuente A, Guillamón E, Villares A, Rostagno MA, Martínez JA. Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. Inflamm Res 2009;58:537–52.
[67] Singh RG, Negi PS, Radha C. Phenolic composition, antioxidant and antimicrobial activities of free and bound phenolic extracts of Moringa oleifera seed flour. J Funct Foods 2013;5:1883–91.
[68] Cushnie TPT, Lamb AJ. Antimicrobial activity of flavonoids. Int J Antimicrob Agents 2005;26:343–56.
[69] Harborne JB, Williams CA. Advances in flavonoid research since 1992. Phytochemistry 2000;55:481–504.