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Research Article

Qualitative and Quantitative Estimation of Flavonoids and Phenolic Compounds and the Biological Activities of *Colvillea racemosa* Cultivated in Egypt

Azza A. Shafei

Pharmacognosy Department, Faculty of Pharmacy, Al Azhar University (Girls), Cairo, Egypt

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ABSTRACT

The phenolic and flavonoid contents of the alcoholic extract of *Colvillea racemosa* were determined using HPLC and colorimetric analysis. Twentythree phenolic components and eleven flavonoidal compounds were detected. E-vanillic and benzoic acid (2489.16 and 454.43 ppm, respectively) were the major phenolic components, while hesperidin and naringin (329.73 and 193.39 ppm, respectively) were the major flavonoids. The colorimetric analysis of phenolic and flavonoidal contents resulted in 36.45 and 66.8 mg/gm respectively. The antimicrobial activity against some gram negative, gram positive bacteria and fungi were compared using ethyl acetate, alcoholic and acetone extracts. The acetone extract showed promising results. The cytotoxic activity was done using 3-(4, 5-dimethylthiosolyl-2)-2, 5-diphenyl tetrazoliam bromide (MTT) assay against colon carcinoma cell lines (HCT-116) on the previously mentioned extracts. The results revealed that alcoholic extract was the most potent one with IC_{50} =4.52µg. Also, the antioxidant property of the alcoholic extract was examined using 2, 2-Diphenyl picrylhydrazyl (DPPH) method, providing IC_{50}= 79.19 ug/ml.

Keywords: *Colvillea racemosa*, Fabaceae, Caesalpinioideae, flavonoids, phenolics, cytotoxic, antimicrobial and antioxidant.

INTRODUCTION

The tree of *Colvillea racemose* is particularly known for its bright orange flowers that grow in a large cone or cylinder shaped clusters. The tree has a small deep green leaves superficially similar to Delonix regia,the tree is native to Madagascar, now widely grown as an ornamental plant[1]. Fabaceae is one of the three largest families of flowering plants, exceeded only by the Compositeae and Orchidaceae. This Family comprises 728 genera and 19325 species, it was divided into three subfamilies; Mimosoideae, Caesalpinioideae and Papilionoideae[2]. Genus Colvillea belongs to the subfamily Caesalpinioideae which includes 171 genera and about 2250 species of tropical, sub-tropical trees and shrubs[3]. Genus Colvillea comprises only one species *Colvillea racemosa*. The family frequently contain alkaloids, proanthocyanidins and flavonoids. Flavonoids constitute one of the most universal group of plant phenolic. In the last few years, the identification and development of phenolic compounds or extracts from different plants have become a major area of health and medical-related research[4]. Previous comprehensive studies proved that the plant polyphenols possess diverse effects on the biological systems[5,6]. The diversity of their structure is the basis of the recent increase in the detection of various biological and pharmacological activities which have been extensively researched such as anti-tumor, anti-bacterial, hepatoprotective, anti-oxidant, anti-allergic, diuretic, anti-diarrheal, anti-inflammatory and antiviral[7,8].

MATERIAL AND METHODS

Plant Material

Leaves of *Colvillea racemosa* were collected in June 2013 from Mohamed Ali museum in Giza, Egypt and were kindly authenticated by Dr. Nabil El Hadidy, professor of botany, faculty of science Cairo University. Voucher specimen (Crl-001) has been deposited in the Faculty of pharmacy, Al Azhar University (Girls), Cairo, Egypt. Sample was stored in a dry place until analyzed.

HPLC analysis of total polyphenols and flavonoids[9]

Onegram dry powder of the leaves of *Colvillea racemosa* was weighed into a 100 ml conical flask then dispersed in 40 ml of 62.5% aqueous methanol. The mixture was then ultrasonicated for 5 min., 10 ml of 6M HCL was added. Hydrolysis was carried out in a water bath at 90º C for 2 hrs. After hydrolysis, the sample was allowed to cool, filtered made up to 100 ml with methanol, and ultrasonicated for 5 min. Before quantification by HPLC, the sample was filtered through a 0.4µm membrane filter into the sampler vial for injection by using HPLC AGILENT 1100 series adopting the following conditions; the column type was ODS column with dimension of 5 µm x4 mm, 350C oven temperature, 0.7 ml/min flow rate, injection volume was 5 µl of the standard and 40 µl of sample extracts and it was equipped with UV detector of 330 nm for flavonoids and 280 nm for polyphenols. The relative concentration of each of the detected polyphenols and flavonoids was determined by the regression equation.

**Author for Correspondence*

Table 1: Total polyphenol compounds

Polyphenolic compounds	Concentration (mg/100gm dried plant powder)
Gallic acid	19.92
Pyrogallol	103.61
Tyrosol	114.59
Sinapinic acid	60.45
Chlorogenic	64.12
Catechol	187.94
Catechin	47.37
Protocatchuic	62.69
Ferulic acid	98.72
P-Coumaric	48.96
Cinnamic acid	11.84
Ellagic acid	232.16
Epicatechin	163.27
Caffeine	64.92
Syringic acid	71.04
Vanillic	28.66
Isoferulic	94.97
E-Vanillic	2489.16
Reversetrol	25.10
Alfa-coumaric	18.77
P-OH-Benzoic	454.43
3,4,5 trimethoxy-cinnamic	37.06
Salicylic	149.17

Table 2: Total flavonoidal compounds

Flavonoids	Concentration (mg/100gm dried plant powder)
Rutin	166.36
Rosmarinic acid	31.76
Quercetrin	18.34
Quercetin	23.45
Naringin	193.39
Hesperidin	329.73
Hespertin	100.74
Kaempferol	12.44
Apigenin	38.74
Narengenin	1.49
7-OH flavone	6.38

Colorimetric estimation of total phenolic content[10]

1 ml of sample (1 mg/ml) was mixed with 1 ml of Folin-Ciocalteu's phenol reagent. After 5 min, 10 ml of a 7% Na2CO3 solution was added to the mixture followed by the addition of 13 ml of deionized distilled water and mixed thoroughly. The mixture was kept in the dark for 90 min at 23°C, after which the absorbance was read at 750 nm. The total phenolic content was determined from extrapolation of calibration curve which was made by preparing gallic acid solution. The estimation of the phenolic compounds was carried out in triplicate. The total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per g of dried sample.

Colorimetric estimation of total flavonoid content[11]

Aluminium chloride colorimetric technique was used for flavonoids estimation. 10 mg of rutin was dissolved in 10 mL of methanol to get 1000 µg/mL solution and was used as standard. Aliquots ranging from 0.01 to 0.08 mL from the above stock solution were taken in different tubes. 1.5 mL of methanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water was added to each tube. The reaction mixture was kept at room temperature for30 min. The absorbance of the resulting solutions was measured at 415 nm against reagent blank. The calibration curve was prepared by plotting absorbance against concentration and it was found to be linear over this concentration range. 10 mg of extracts were dissolved in 10 mL of methanol to get 1 mg/mL solutions. The concentration of total flavonoid in the test sample was determined from the calibration curve. The total flavonoid content in the extract was expressed as rutin equivalent (mg RE/g extract).

Antimicrobial activity

The antimicrobial study of (alcoholic, ethyl acetate and acetone extracts) was evaluated against *Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumonia, Salmonella typhimurium, Aspergillus fumigatus, Penicillium italcum, Syncephalastrum racemosum* and *Candida albicans* using the following methods:

Sensitivity tests by Kirby-Bauer method (disc diffusion method)

Antimicrobial activity of the tested samples was determined using a modified Kirby-Bauer disc diffusion method [(12)]. 100 µl of the test bacteria/fungi were grown in 10 ml of fresh media until they reached a count of approximately 108 cells/ml for bacteria or 105 cells/ml for fungi [(13)]. 100 µl of microbial suspension was spread onto agar plates corresponding to the broth in which they were maintained. Standard discs of ampicillin, gentamycin (antibacterial agents) and amphotericin B (antifungal agent) served as positive controls for antimicrobial activity while filter discs impregnated with 10 µl of solvent (DMSO) were used as a negative control. Blank paper discs (Schleicher & Schuell, Spain) with a diameter of 8.0 mm were impregnated with 10µl of tested concentration of the stock solutions.

MIC determination using agar dilution method

Standardized bacterial suspensions were prepared to a final cell density of 6 x 10^5 CFU/ml (colony forming units/ml). Serial dilutions from the methanolic plant extract (0 – 320 µg / ml) were prepared and mixed with 5 ml of the standardized bacteria suspension then added to the plates and incubated for 24 h at 37 °C. CFU were counted for each dilution[14].

Cytotoxic activity

The cytotoxic activity was examine on (alcoholic, ethyl acetate and acetone extracts) using Colon carcinoma cell line (HCT-116) by MTT assay[15].

Metabolic status assessment of cultured cancer cells in the absence or presence

Colvillea racemosa alcoholic extract was carried out by performing tetrazolium salt-based cell viability assays. Briefly, cells were plated in 96-well plates at a density yielding 80–90% confluence when the cytotoxicity assay

Table 3: Antimicrobial activity of different extracts of *Colvillea racemosa* subfamily Caesalpinioideae

Microorganisms	Type	Inhibition zone diameter (mm/mg sample)					
		Standard antimicrobial agents			*Colvillea racemosa* subfamily Caesalpinioideae		
		Ampicilin antibacterial ($G^{\pm}$)agent	Gentamycin antibacterial (G^{-})agent	Amphotericin B (antifungal agent)	Alcoholic extract	Ethyl acetate extract	Acetone extract
Staphylococcus aureus	G^{+}	22.9 ± 0.14	--------	--------	17.2 ± 0.44	13.6 ± 0.63	19.6±0.58
Bacillus subtilis	G^{+}	28.3 ± 0.37	--------	--------	16.3 ± 0.58	13.9 ± 0.25	20.9 ± o.25
Klebsiella pneumoniae	G^{-}	--------	26.25 ± 0.25	--------	14.3 ± 0.37	10.2 ± 0.58	18.6 ± 0.58
Salmonella typhimurium	G^{-}	--------	25.32 ± 0.63	--------	16.2 ± 0.25	12.6 ± 0.44	20.3 ± 0.25
Aspergillus fumigatus	Fungus	--------	--------	22.9 ± 0.44	18.3 ± 0.44	NA	22.6 ± 0.44
Penicillium italcum	Fungus	--------	--------	21.3 ± 0.37	15.4 ± 0.58	NA	21.9 ± 0.19
Syncephalastrum racemosum	Fungus	--------	--------	19.5 ± 0.55	17.9 ± 0.37	NA	20.9 ± 0.44
Candida albicans	Yeast	--------	--------	21.4 ± 0.25	13.2 ± 0.25	NA	16.8 ± 0.25

Data are expressed in form of mean ± standard deviation
*$G^{\pm}$ = Gram positive bacteria. *G^{-} = Gram negative bacteria. *NA= No Activity.

was performed. Complete medium was refreshed and eight replicates cultures were treated with different doses of the extracts for 72 h. Control samples were treated with equivalent amounts (v/v) of solvent (DMSO). Cell viability was measured by the bioreduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium-bromide (MTT) to a colored formazan product that was dissolved in 100 µL of DMSO and measured at 570 nm with a microplate reader. The optical density obtained was directly correlated with cell quantity. All the results corresponding to MTT experiments were expressed as the mean of a minimum of six to eight replicates.

Antioxidant activity

Freshly prepared (0.004%w/v) methanol solution of DPPH radical was prepared and stored at 10°C in the dark. methanol solution of the test compound was prepared. A 40 uL aliquot of the methanol solution was added to 3ml of DPP solution. Absorbance measurements were recorded immediately with a UV-visible spectrophotometer (Milton Roy, Spectronic 1201). The decrease in absorbance at 515 nm was determined continuously, with data being recorded at 1 min intervals until the absorbance stabilized (16 min). The absorbance of the DPPH radical without antioxidant (control) and the reference compound ascorbic acid were also measured. All the determinations were performed in three replicates and averaged. The percentage inhibition (PI) of the DPPH radical was calculated according to the formula: PI = [{(*A*C- *A*T)/ *A*C} x 100] Where *A*C = Absorbance of the control at t = 0 min and *A*T = absorbance of the sample +DPPH at t = 16 min[16].

RESULTS AND DISCUSSION

HPLC analysis of total polyphenols and flavonoids

The total concentrations of polyphenolic and flavonoidal compounds in the methanolic extract are shown in table1 and 2 respectivily. Results in table 1 showed the presence of 23 phenolic compounds in which E-vanillic and p-OH benzoic are of highest concentrations (2489.16 and 454.43mg/100gm respectively). While in table 2 hesperidin and naringin were the major flavonoids among the eleven detected compounds with concentrations (329.73 and 193.39 mg/100gm respectively).

Coloremetric estimation of phenolic and flavonoid contents

Total phenolic and flavonoid contents of alcoholic extract of *Colvillea racemosa* were estimated calorimetrically. The total phenolic content was (36.45mg/gm) expressed as gallic acid equivalent per gram. While the contents of total flavonoid (66.8 mg/gm) were expressed in terms of rutin equivalent.

Antimicrobial activity

The antimicrobial activity of different extracts is illustrated in table 3, the acetone extract showed promising results. Minimum inhibitory concentration (MIC)of acetone extract are shown in table 4 against *Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumonia, Salmonella typhimurium, Aspergillus fumigatus, Penicillium italcum, Syncephalastrum racemosum* and *Candida albicans* revealing values 3.9, 1.95, 3.9, 1.95, 0.98, 0.98, 1.95 and 15.63µg/ml respectively. On comparing the results of MIC of the acetone extract with the standard anti-fungal amphotericin B, we concluded that the acetone extract has the same antifungal activity on

Table 4: Minimum Inhibitory Concentrations of *Colvillea racemosa* subfamily Caesalpinioideae (Acetone extract) against different microorganisms

Microorganisms	Type	Minimum Inhibitory Concentration (MIC) ug/ml			
		Standard antimicrobial agents			*Colvillea racemosa* subfamily Caesalpinioideae (Acetone extract)
		Ampicillin antibacterial ($G^{\pm}$) agent	Gentamycin antibacterial (G^{-}) agent	Amphotericin B (antifungal agent)	
Staphylococcus aureus	G^{+}	0.98	--------	--------	3.9
Bacillus subtilis	G^{+}	0.24	--------	--------	1.95
Klebsiella pneumoniae	G^{-}	--------	0.49	--------	3.9
Salmonella typhimurium	G^{-}	--------	0.49	--------	1.95
Aspergillus fumigatus	Fungus	--------	--------	0.98	0.98
Penicillium italcum	Fungus	--------	--------	1.95	0.98
Syncephalastrum racemosum	Fungus	--------	--------	3.9	1.95
Candida albicans	Yeast	--------	--------	1.95	15.63

Aspergillus fumigatus as amphotericin B with MIC (0.98) for both. While the acetone extract has a higher antifungal activity against *Syncephalastrum racemosum* (1.95) over the amphtericin **B** (3.9). Flavonoid and Phenolic compounds were reported to have antibacterial activities[17-20]. Antifungal activity was found due to the presence of flavonoid and phenolic compounds[21-24]. Both phenolic and flavonoidal compounds were proven to be present in *Colvillea racemosa.*

Cytotoxic activity

The three pre-mentioned extracts of *Colvillea racemosa* showed dose dependent cytotoxic activity against colon carcinoma cell line (HCT-116). The alcoholic extract showed promising cytotoxic activity with IC_{50} = 4.52 ug. While the ethyl acetate extract showed a moderate cytotoxic activity with IC_{50} = 6.4 ug. The lowest cytotoxic activity was shown with the acetone extract who has IC_{50} = 12.5ug. The cytotoxic activity could be attributed to the presence of phenolic and flavonoid contents which are the most frequent constituents in *Colvillea racemosa* alcoholic extract[15,25,26].

Antioxidant activity

The free radical scavenging activity of the alcoholic extract of *Colvillea racemosa*, based on the scavenging activity of the stable 1, 1-diphenyl-2- picrylhydrazyl (DPPH) showed antioxidant activity with IC_{50}= 79.19µg/ml expressed in terms of ascorbic acid equivalents (AAE). The use of natural antioxidants from plants is medically helpful and least detrimental with very few side effects as compared to synthetic ones. As indicated by various studies, the beneficial effects of antioxidant have been attributed to the Secondary metabolites such as phenolics and flavonoids[27-30].

CONCLUSION

On the basis of the results obtained in the present study, it was concluded that the alcoholic leaf extract of *Colvillea racemosa* which contains large amount of phenolic and flavonoid compounds, exhibit high cytotoxic activity against colon carcinoma cell line HCT-116 and antioxidant activity. The acetone extract of *Colvillea racemosa* has a promising antifungal activity against both *Aspergillus fumigates* and *Syncephalastrum racemosum*. Hence, more queries will be addressed in future studies targeting isolation of bioactive compounds responsible for such activities.

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