Research Article

GCMS Analysis, Determination of Total Phenolics, Flavonoid Content and Free Radical Scavenging Activities of Crude Extracts of *Plumeria rubra* Leaves

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ABSTRACT

Objective: To perform phytochemical screening, estimate total Phenolics, flavonoid contents and to evaluate Antioxidant potential of *Plumeria rubra* leaves extracts.

Methods: The dried powdered leaves of *Plumeria rubra* were extracted exhaustively by Soxhlet extraction with Pet ether, Chloroform, Methanol with increasing order of polarity. Folin cio calteau and Aluminium chloride method were used for determination of total phenol and flavonoid content in leaves extracts. Methanol extracts were analyzed by GCMS to identify and characterized the chemical compound present in crude extract. Hydrogen peroxide, 1, 1 DPPH were used to determine antioxidant activity.

Results: Phytochemical analysis of Methanol extract showed presence of major classes of phytochemicals. Methanol extract was found the highest phenolic and flavonoid content. Gas chromatography and Mass spectroscopy revealed presence of 23 phytoconstituents in Methanol extracts. Methanol extracts show significant antioxidant activity when compared with other Extracts and reference standard as ascorbic acid.

Conclusion: Methanol extract shows that leaves *P.rubra* rich source of Phenolic and flavonoid content that play important role in prevention of many diseases.

Keywords: *Plumeria rubra*, antioxidant, flavonoid, phenols, GCMS.

INTRODUCTION

Plumeria is genus of lactiferous trees and shrubs. Native of tropical America, some Ornamental species are grown in warmer region of world. About eight species are reported. From India, but owing to the overlapping character of some species, it become difficult to fix Their identity. In traditional system of medicine of India *Plumeria* species are widely used as purgative, remedy for diarrhea, cure of itch, bronchitis, cough, asthma, fever, piles, dysentery, blood disorder and tumors.

Plumeria is indigenous to tropical America and is found from southern Mexico to northern South America and also most abundant in India. However, due to its ease of propagation. Through cutting, many species and hybrids of *Plumeria* are now widely cultivated and Distributed in the warmer region of world.

The trees were introduced to Malaysia and at least three main species are commonly found *Plumeria obtusa*, *Plumeria rubra* and *Plumeria acuminate Plumeria rubra* commonly grown for their ornamental purpose. The plant is propagated through cuttings. It sets seed rarely in India. The plants raised from the seed shown a wide Variation in character evidently being different strains. *Plumeria rubra* is small tree 3.5-6.0 m in high, commonly grown in gardens, leaves Lanceolate to obovate–oblong. Flowers very Fragrant, generally red pink or purple center rich with yellow.

Flowers large in terminal 2-3 chotomous cymes, bracts many broad, deciduous, calyx small, Glandular within, lobes broad, obtuse. Corolla salver shaped, throat necked lobes overlapping to the lefty, rarely to right. Stamen near base of tube. Seeds oblong or Lanceolate. Its broad, usually round-headed canopy is often about as wide as the tree is tall.[1]

Traditional uses:

Fruit is reported to be eaten in West Indies. India however it has been used as abortifacient.

Leaves-Anti-inflammatory, purgative, Antibacterial, Anthelmintic

Root bark- Drastic, Purgative, Blennorrhagia.

Latex – Used in toothache and for carious teeth.

Flowers- Aromatic, Bechic and used as very popular pectoral Syrup.[2]

Therefore, it is worthwhile to screen the Methanolic extract of *P.rubra* leaves for the presence of phytochemicals, to identify and characterize phytoconstituents in its various crude extracts for chemical profiling by gas chromatography-mass spectrometric (GC-MS) analytic technique and to evaluate its antioxidant potential by using in vitro methods to correlate with the phenolics and flavonoid content.

MATERIALS AND METHODS

Chemicals

Rutin, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and Gallic acid were purchased from Sigma-Aldrich, USA. Folin- Ciocalteu reagent was obtained from Merck, Germany. All other chemicals and solvents used in the study were of analytical grade procured locally.

Collection of plant material

P.rubra leaves were collected in June 2016 from Nashik region in India and authenticated by a botanist from BSI Pune. The leaves were washed thoroughly with water to remove dust and dried under the shade at room temperature the dried leaves were ground using kitchen blender to obtain the course powder and kept in an air tight container till further use

Preparation of Extracts

The dried powdered leaves were exhaustively extracted with continuous hot Soxhlet extraction using solvent Pet Ether, Chloroform and Methanol.

The freshly prepared crude extracts of leaves were subjected to qualitative chemical tests to identify various classes of bioactive chemical constituents present in the leaves using standard procedures [3]

Determination of total phenols by Folin-Ciocalteu reagent method

Gallic acid Stock Solution: In a 100 ml volumetric flask, dissolved 50mg of dry Gallic acid in 10 ml of Methanol and diluted to volume with water.

Preparation of stock solution of extracts: A stock solutions of 1000µg/ml of each extract was prepared in Methanol.

Sodium carbonate solution: Sodium carbonate (200 gm.) was dissolved at 70 - 80 ^{o}C and volume was made with distilled water up to 1 liter. It was filtered through glass wool and allowed to stand overnight.

Principle:

The Folins reagent is sensitive to reducing compounds, polyphenols there by producing blue colored complex. The quantative phenolics estimation was performed at max. 765 nm by change in intensity of Folin-phenolic compounds complex. [4]

Method:

Preparation of calibration curve:

The phenolics content was determined according to the method described by Spanos with slight modifications. To prepare a calibration curve 0.5, 1, 2, 3, 4 and 5 mL of the Gallic acid stock solution was transferred to 10 ml volumetric flasks, and then diluted to volume with water to produce Gallic acid solutions having concentrations of 25, 50, 100, 150, 200, and 250 µg/ ml, the effective range of the assay.

1 ml of standard Gallic acid solution from each dilution was taken in 25 ml volumetric flask, added 10 ml of water, 1.5 ml of Folin-Ciocalteu Reagent and allowed to stand for 10 min. then 4 ml of sodium carbonate solution was added in each volumetric flask and volume was adjusted with distilled water. Readings were taken after 1 hr at 765 nm by spectrophotometer against reagent blank. The calibration curve of absorbance Vs. Concentration was plotted. [5]

Estimation of Phenol Content**:** 1ml of stock solution of extracts was transferred in 25 ml volumetric flask; similar procedure was adopted as above described in preparation of calibration curve. With the help of calibration curve, the phenolic concentration of extracts was determined.

Estimation of total flavonoid content (TFC) by aluminum chloride colorimetric method

Rutin Stock Solution: In a 50mL volumetric flask, dissolved 10mg of Rutin in methanol and made volume with Methanol.

Preparation of stock solution of extracts: A stock solution of 1000µg/ml of each extract was prepared in methanol.

Aluminium chloride solution: 10g of Aluminium chloride added in 85 mL water with continuous stirring, cool it and volume was made up to 100mL with water.

Potassium acetate solution: 5g of Potassium acetate dissolved in water and made volume up to 100mL with water.

Principle: The TF content of the *Plumeria rubra* extract was determined using the Aluminium chloride colorimetric method. The Aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. Aluminium chloride also forms acid labile complexes with the ortho - dihydroxyl groups in the A- or B-ring of flavonoids [6]

Preparation of calibration curve:

To prepare a calibration curve 0.5, 1, 2, 3, 4 and 5 mL of the Rutin stock solution was transferred to 10 mL volumetric flasks, and then diluted to volume with water to produce Gallic acid solutions having concentrations of 10, 20, 40, 60, 80, and 100 µg/ mL, the effective range of the assay.

The flavonoids content was determined according to the method described by Kumaran and Karunakaran. Each dilution of standard Rutin solution (10 to 100 µg/ml) taken separately in test tubes. To each test tube 1.5ml methanol, 0.1ml 10% Aluminium chloride solution, 0.1ml 5 % potassium acetate solution and 2.8 ml distilled water were added and mixed well. Sample blank for all the dilution of standard Rutin and all extract were prepared in similar manner by replacing Aluminium chloride solution with distilled water. All the prepared solutions were filtered through Whatmann filter paper before measuring their absorbance. Absorbance was taken at 415 nm against the suitable blank.

Estimation of flavonoids content: Total flavonoid content was determined using 0.5ml of each extract stock solution (1000µg/ml) was transferred test tube; similar procedure was adopted as above described in preparation of calibration curve. The amount of flavonoid in the extract was determined from calibration curve of Rutin.

Antioxidant activity by Hydrogen peroxide scavenging method

Principle: Scavenging of H_2O_2 by extracts may be attributed to their Phenolics which can donate electrons to H_2O_2, thus neutralizing it to water. [7]

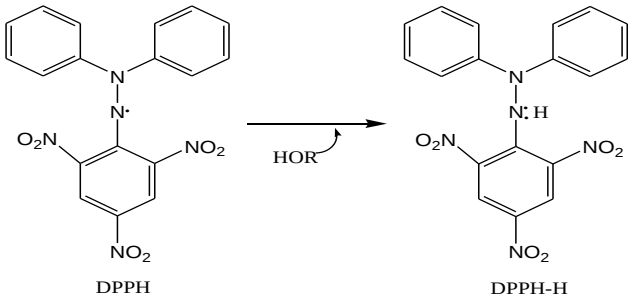
Procedure: The hydroxyl radical scavenging activity of Leavesof *P.rubra* extracts was measured by the method of Xiao. A hydrogen peroxide solution (40 mM) was prepared in phosphate buffer (pH 7.4). Different concentration of extracts was prepared in methanol. In that 0.6 mL hydrogen peroxide solution was added. The absorbance of various dilution was determined after ten minutes against a blank solution containing phosphate buffer at 230 nm.[8]

% inhibition calculated as per above formula & IC_{50} was calculated.

Antioxidant activity by DPPH Free Radical Scavenging method

Principle: DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of Phyto-constituents.

DPPH is nitrogen centered free radical. It reacts similar as peroxyl radial. The reaction rate directly correlates with antioxidant activity. The odd electron in DPPH free radical gives a strong absorption maximum at 517nm and is purple in color. The color turn from purple to yellow as the molar absorptivity of DPPH radical at 517nm reduces when odd electron of DPPH radical becomes paired with hydrogen from free radical scavenging antioxidant to form the reduced DPPH-H. The resulting discoloration is stoichiometric with respect to number of electrons captured. [9]



DPPH DPPH-H

Procedure: Various concentrations of each *P.rubra* leaves extract were prepared. In each above 4mL extract solution, 100µl of DPPH solution (13mg/Lit.) was added. 100 µl of DPPH solution in 4 mL methanol was treated as blank. Ascorbic acid was used as standard. Absorbance of each solution was taken after 15min at 517nm. % inhibition calculated as per above formula & IC_{50} calculated [10]

RESULTS AND DISCUSSION

Yield of Extracts Obtained After Continues Solvent Soxhlet Extraction

Table 1: Percent of extract of Leaves

Extracts	Colour of Extract	% (w/w) of Extract obtained
Petroleum ether	Green sticky	7.9 ± 0.13
Chloroform	Dark brown	5.90 ±0.28
Methanol	Dark Brown	6.53 ± 0.43

Phytochemical Tests of Extracts

Table 2: Phytochemical Tests of Extracts

Sr No.	TESTS	Extracts		
		Pet. Ether	Chloroform	Methanol
1.	Test for Alkaloids	–	+	+
2.	Test for Cardiac glycosides	+	_	_
3.	Test for Naphthoquinones	–	–	–
4.	Test for Anthraquinones	–	–	–
5.	Test For Iridodial glycosides	–	–	+
6.	Test for Coumarin glycosides	–	–	–
7.	Test for Cyanogenetic glycosides	–	–	–
8.	Test for Sterols	+	+	+
9.	Test for Triterpenoids	+	+	+
10.	Test for Flavonoids	–	–	+
11.	Test for Tannins	–	–	+
12.	Tests for Carotenoids	+	–	–
13.	Test for Proteins	–	+	+
14.	Test for Carbohydrates	–	–	+

GCMS Profiling of Methanol Extracts

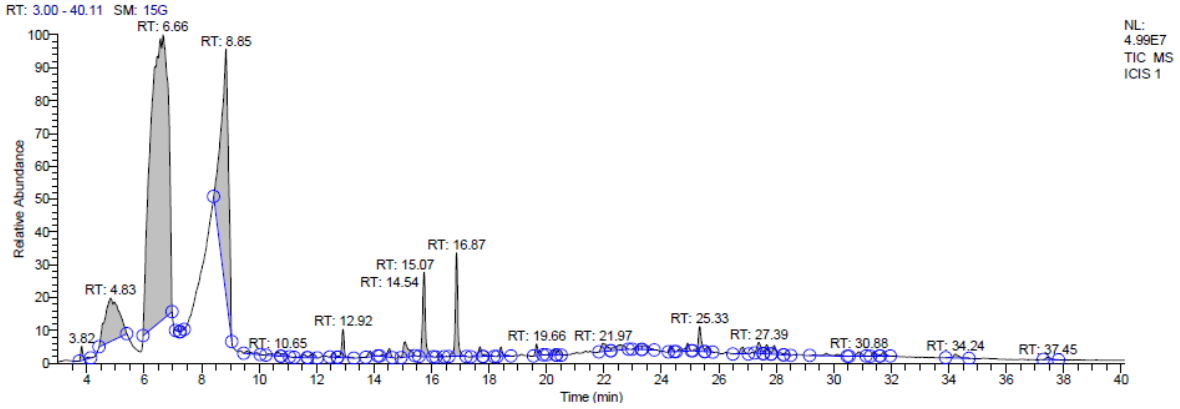


Figure 1: GCMS of Methanol Extract

Table 3: GCMS Profiling of Methanol Extracts

Sr. no	Retention time (Min.)	Name of compound	Molecular Formula
1	6.66	1 Propene 2Methyl	C_4H_8
2	9.58	Formic acid, Butyl esters	$C_5H_{10}O_2$
3	10.65	Pentanoic acid ,3Hydroxy methyl ester	$C_6H_{20}O_3$
4	10.83	Butanoic acid 3methyl –butyl ester	$C_9H_{18}O_2$
5	11.57	Propanoic acid,2Methyl propyl ester	$C_8H_{16}O_2$
6	12.92	Propanoic acid ,2 Methyl	$C_{12}H_{24}O_3$
7	15.75	1-1Dibutoxyisobutane	$C_{12}H_{26}O_2$
8	22.53	Adipic acid,bis(3,3,5 Trimethylhexyl)ester	$C_{24}H_{46}O_4$
9	23.41	a-d Mannofuranoside, methyl	$C_7H_{14}O_6$
10	24.91	Hexadeconoic acid, methyl ester	$C_{17}H_{34}O_2$
11	25.33	N Hexadecanoic acid	$C_{16}H_{32}O_2$
12	26.84	6-Octadecanoic acid, Methyl ester (z)	$C_{19}H_{36}O_2$
13	27.39	Cis 9Hexadeccanal	$C_{16}H_{30}O$
14	27.67	Eicosanoic acid	$C_{20}H_{40}O_2$
15	27.93	Hexadecanoic acid butyl ester	$C_{20}H_{40}O_2$
16	29.74	Eicosane 7hexyl	$C_{26}H_{54}$
17	31.73	Tetradecane ,2,6,10 Trimethyl	$C_{17}H_{36}$
18	34.24	Nonadecane,2methyl	$C_{20}H_{42}$
19	30.88	6Octadecanoic acid, (z)	$C_{18}H_{34}O_2$
20	31.39	Octadecanoic acid, butyl ester	$C_{22}H_{44}O_2$
21	37.45	10-Methyl non adecane/eicosane	$C_{20}H_{42}$
22	27.67	Tetradecanoic acid	$C_{14}H_{28}O_2$
23	26.84	Cis vaccenic acid	$C_{18}H_{34}O_2$

Total Phenol Content

Table 4: Total Phenol content of Extracts

Sample (1000µg/ ml)	Absorbance	Total phenol (mg Gallic acid equivalent/g of dried extract)
Pet Ether Extracts	Nil	Nil
Chloroform Extracts	0.136±0.0005	27.66±0.126
Methanol Extracts	0.285±0.003	64.83± 0.877

Total Flavonoid Content

Table 5: Total flavonoid content of Extracts

Sample (1000µg/ ml)	Absorbance	Total flavonoid (mg Rutin equivalent/g of dried extract)
Pet ether Extract	Nil	Nil
Chloroform Extract	0.148±0.05	18.166±0.007
Methanol Extract	0.227±0.01	28.041±1.371

Antioxidant Activity by Hydrogen Peroxide Method

Table 6: Antioxidant Activity of Extracts by Hydrogen peroxide Method

Conc (µg/ml)	Pet ether extract	Chloroform extract	Methanol extract	Conc. of std(µg/ml)	Ascorbic acid
25	12.30±0.965	11.26±1.31	15.65±1.234	5	20.98±1.32
50	23.53±2.62	22.47±1.98	26.58±2.654	10	41.21±2.04
75	25.18±1.79	32.23±2.217	38.42±1.543	15	59.35±1.456
100	32.69±1.629	46.22±1.73	51.23±2.765	20	75.45±1.65
125	42.36±1.96	53.16±1.698	62.23±2.543	25	98.23±1.810
IC_{50}	203.74	114.411	99.78	Ic 50	12.606

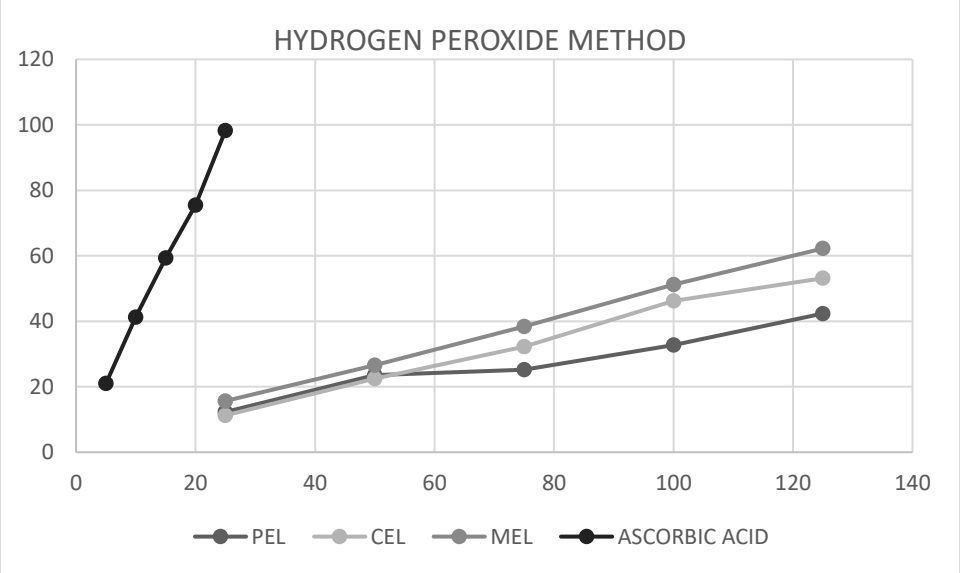


Figure 2: Antioxidant activity of Extracts by Hydrogen Peroxide method

Antioxidant activity By DPPH method

Table 7: Antioxidant Activity of Extract by DPPH method

Conc (µg/ml)	Pet ether leaves extract	Chloroform leaves extract	Methanol leaves extract	Conc of std (µg/ml)	Ascorbic acid
25	12.79±2.03	9.23±2.351	29.00±1.094	5	15.62±1.95
50	23.51±1.5692	20.78±1.895	45.79±5.5834	10	34.28±1.30
75	33.72±2.09	29.32±2.613	52.92±1.86	15	43.52±2.02
100	44.05±1.255	40.55±3.125	64.31±.3818	20	59.65±1.69
125	48.86±1.74	47.56±2.045	68.09±2.020	25	79.85±1.40
IC_{50}	128.98	121.48	69.92	IC_{50}	16.11

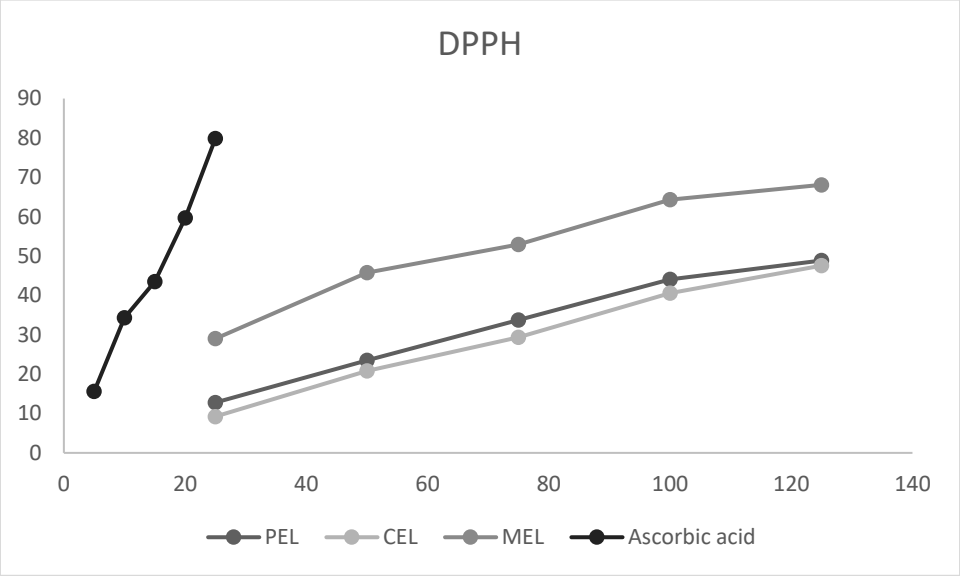


Figure 3: Antioxidant Activity of Extracts by DPPH method

DISCUSSION

Plants contain numerous phytochemical constituents, many of which are known to be biologically active compounds and are responsible for exhibiting diverse pharmacological activities.[11] Some of these secondary metabolites of plants are important source of natural antioxidants that are preferred over synthetic ones because of safety concerns.[12] The bioactive secondary metabolites have been shown to reduce the risk and progression of diseases such as cancer, cardiovascular, neurodegenerative diseases, etc. by scavenging free radicals through various biological mechanisms.

The results of preliminary chemical testing confirmed the presence of various classes of bioactive chemical constituents in ethanolic extract of *P rubra* leaves including polyphenols (tannins and flavonoids), steroids, alkaloid, carbohydrate glycosides, cardiac glycosides, and terpenoid. Numerous reports available on phenolic compounds have demonstrated their usefulness in exhibiting potential biological activities such as antioxidant, antidiabetic, hepatoprotective, anti-inflammatory, antimicrobial, anticancer etc.[13,14] The antioxidant activity of phenolic compounds is mainly due to their reduced properties which allow them to act as metal chelators, absorb and neutralize free radicals.[15] Flavonoids and tannins are considered to be the most promising polyphenolic compounds among plant secondary metabolites.[16]

Therefore, based on the phytochemical screening results, the total phenolic and flavonoid contents of different extracts of *P.rubra* leaves were estimated and also its antioxidant potential were investigated by in vitro DPPH and H_2O_2 assay methods

The results of GC-MS and preliminary photochemical testing indicated that *P.rubra* leaves contained numerous bioactive phytoconstituents belonging to various classes

The leaves extract upon quantification by colorimetric methods were found to be rich in phenolic compounds (tannins and flavonoids) and therefore exhibited very good scavenging activity against DPPH and H_2O_2 free radicals. The antioxidant activity by both methods was concentration dependent and comparable to standard antioxidant, ascorbic acid. Based on the results, it can be concluded that *P.rubra* leaves could be used as a natural source of antioxidants and its regular consumption in diet could provide health benefits to humans by the protection against oxidative stress. Further detailed in vitro and in vivo correlation studies along with isolation of active constituents are needed to unravel novel treatment strategies for free radical induced diseases.

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