RESEARCH PAPER

COMPARATIVE PHYTOCHEMICAL PROFILING AND *IN VITRO* ANTIOXIDANT AND ACETYLCHOLINESTERASE INHIBITORY ACTIVITIES OF CHLOROFORM, ETHYL ACETATE, AND METHANOLIC ROOT EXTRACTS OF *MIRABILIS JALAPA*

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ABSTRACT

Background: *Mirabilis jalapa* (*Nyctaginaceae*) is a widely distributed ornamental and ethnomedicinal plant, the tuberous root of which has been traditionally used for wound healing, antiviral, anti-inflammatory purposes and is the source of the well-characterized ribosome-inactivating antiviral protein MAP. However, comparative solvent-dependent phytochemical and bioactivity profiles of *M. jalapa* roots remain to be explored. **Objective:** The aim of this study was to sequentially extract *M. jalapa* roots using solvents of increasing polarity (petroleum ether, chloroform, ethyl acetate and methanol) and to evaluate the physicochemical parameters, total phenolic (TPC), flavonoid (TFC) and alkaloid (TAC) contents and *in vitro* antioxidant (DPPH) and acetylcholinesterase inhibitory (Ellman's method) activities of the chloroform, ethyl acetate and methanolic root extracts. **Methods:** Standard pharmacognostic and phytochemical analyses were performed, and the IC_{50} values for antioxidant and acetylcholinesterase inhibitory activities were calculated from the corresponding dose–response curves. **Results:** The methanolic extract showed the highest TPC (175.8 ± 1.52 mg GAE/g), TFC (35.18 ± 0.48 mg QE/g), TAC (0.47± 0.026 mg AE/g) and the strongest DPPH radical scavenging (IC_{50} = 276.87 ± 0.39 µg/mL) and acetylcholinesterase inhibitory activity (IC_{50} = 76.99 ± 0.36 µg/mL) among the three extracts, in the order of methanol, ethyl acetate and chloroform. **Conclusion:** The results suggest that extraction with polar solvents, particularly methanol, is more effective for recovering constituents associated with antioxidant and acetylcholinesterase inhibitory activities from *M. jalapa* roots. These findings support further investigation of *M. jalapa* roots as a potential source of natural bioactive compounds relevant to oxidative stress and cholinergic dysfunction

Keywords: *Mirabilis jalapa*; sequential extraction; total phenolic content; DPPH; acetylcholinesterase inhibition; antioxidant

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.INTRODUCTION

Mirabilis jalapa (Four O'clock plant) is a perennial herbaceous ornamental plant belonging to the family *Nyctaginaceae*. It is cultivated throughout tropical and subtropical regions and has a long history of use in traditional and folk medicine systems, where different plant parts are used for the management of inflammation, wounds, infections and diabetes[1-7]. The tuberous root, one of its organs, has been the focus of

research, not only because of its traditional use for the treatment of wounds, but also due to the presence of the Mirabilis antiviral protein (MAP), a type I ribosome-inactivating protein that has been well characterized at the molecular and structural level[8-9].

Phytochemical studies of the roots have led to the identification of a number of constituents, including the rotenoids (mirabijalones A–D and related boeravinones), the pyridine alkaloid trigonelline, the β-carboline alkaloid flazin, sterols such as β-sitosterol and daucosterol, and various phenolic and flavonoid compounds. These constituents have been reported to possess antioxidant, anti-inflammatory, antibacterial, wound-healing, and antiviral effects[10-14]. Most pharmacological studies, however, have focused on single-solvent extraction, commonly using methanol or ethanol, whereas comparative investigations of the solvent-polarity-dependent phytochemical composition and bioactivities of *M. jalapa* roots remain limited.

Sequential extraction using solvents of increasing polarity is a commonly employed approach in pharmacognosy for the differential extraction of plant constituents according to their physicochemical properties and for identifying the solvent fraction most effective in recovering bioactive compounds of interest. In the present study, the root powder of *M. jalapa* was successively extracted with petroleum ether, chloroform, ethyl acetate and methanol. The chloroform, ethyl acetate and methanolic extracts were screened for total phenolic, flavonoid and alkaloid content and *in vitro* antioxidant (DPPH radical scavenging) and acetylcholinesterase inhibitory (Ellman's method) activity. Acetylcholinesterase inhibitory activity was selected for evaluation because of the continued interest in plant-derived acetylcholinesterase inhibitors as potential leads for the symptomatic management of Alzheimer's disease. Several plant-derived alkaloids and phenolic compounds have demonstrated promising *in vitro* acetylcholinesterase inhibitory activity, highlighting the potential of medicinal plants as sources of acetylcholinesterase-inhibitory compounds.

The present study therefore aims to (i) determine the physicochemical parameters of *M. jalapa* root powder, (ii) quantify total phenolic, flavonoid and alkaloid content of chloroform, ethyl acetate and methanolic root extracts and (iii) evaluate *in vitro* antioxidant and acetylcholinesterase inhibitory potential of all the three extracts of *M. jalapa* roots.

MATERIAL AND METHODS

Collection and Authentication of plant material: *M. jalapa* roots were collected from local area of Sundargarh district, Odisha, India and identified by experts in Botany Department of Sambalpur University.

Chemicals: Gallic acid, quercetin, ascorbic acid, atropine, DPPH, were purchased from Himedia, Mumbai. Folin–Ciocalteu reagent, aluminum chloride and bromo cresol green were procured from Molychem, Mumbai. Acetylcholinesterase (AChE) 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), and acetylthiocholine iodide were purchased from Sigma. All the chemicals used in the study were of analytical grade.

Physico-Chemical Evaluation: The root powder of *M. jalapa* was evaluated for physico-chemical parameters such as total ash value, acid insoluble ash value, water soluble ash value and extractive value (alcohol soluble and water soluble) as per WHO guidelines[15].

Extraction of plant material: The freshly collected roots of *M. jalapa* were washed thoroughly with water to remove the adhering soil, cut into small pieces, shade dried, coarsely powdered and stored in air tight container till use. The powdered root material was successively extracted using a Soxhlet apparatus with solvents of increasing polarity in the following sequence: petroleum ether, chloroform, ethyl acetate, and methanol. The marc obtained after each extraction was air-dried before being subjected to extraction with the next solvent. Each extract was filtered and concentrated under reduced pressure by means of a rotary evaporator. Further the extracts having semisolid mass were kept in desiccator[15]. The petroleum ether fraction was used for defatting the material and not analyzed further in this study. The chloroform, ethyl acetate and methanolic fractions were taken forward for phytochemical analysis and bioactivity evaluation.

Preliminary phytochemical study: Preliminary phytochemical study was carried out to identify the presence of different phytoconstituents in all three extracts of *M. jalapa* roots [16].

Estimation of total phenolic contents by colorimetric method

Total phenolic content of the plant extracts was measured by colorimetric technique. 1ml (1000µg/ml) plant extract solution was combined with 1.5ml of Folin-Ciocalteu (FC) reagent followed by incubation at room temperature for 5min. The reaction mixture was further incubated at room temperature for 30 min after the addition of 4 ml (7.5%) sodium carbonate and 3.5 ml of distilled water. Absorbance was measured at 765 nm using a UV-Vis spectrophotometer. Standard graph was plotted with concentration of 20-100µg/ml of gallic acid and total phenolic content was estimated as mg gallic acid equivalents (GAE) per gram of dry weight of extract[17].

Estimation of total flavonoid content by colorimetric method

The total flavonoid content in the plant extract was quantified using the colorimetric technique. The extract (1ml, 1000µg/ml) was mixed with 3ml methanol, 0.2ml (10%) aluminum chloride, 0.2ml (1M) potassium acetate and 5.6ml distilled water, and incubated at room temperature for 30 min. Absorbance was measured at 415 nm using UV-Vis spectrophotometer after incubation. The standard graph was plotted using 10-50µg/ml concentration of quercetin and the total flavonoid content was calculated and reported as mg quercetin equivalents (QE) per gram of dry weight of extract[18].

Estimation of total alkaloid content by colorimetric method

Plant extract 1 g was dissolved in 2N HCl, and subsequently filtered. 1 mL of this solution was taken and transferred into separatory funnel and washed with 30 ml of chloroform, and pH of solution was adjusted to neutral using 0.1 N NaOH following this, 5 mL of bromocresol green and 5 mL of phosphate buffer (pH 4.7) were added to the solution. The mixture was shaken, resulting in the formation of a yellow complex, which was then extracted with 1, 2, 3 and 4 mL chloroform through vigorous shaking in the separatory funnel. The extracts were collected in 10 mL volumetric flask and diluted to 10 mL with chloroform. For plotting the standard graph atropine was used as standard and 100 µg/ml standard stock solution was prepared in distilled water. From this stock solution, using aliquots (0.2, 0.4.0.6, 0.8 and 1.0ml) was transferred to the separating funnels and the same procedure was repeated like plant extract. The absorbance of both test and standard solutions was measured at 470 nm using UV–Vis spectrophotometer. The results were expressed as mg atropine equivalents (AE) per gram of dried extract, based on an atropine standard calibration curve[19-20].

***In vitro* antioxidant activity by DPPH method**

A 2 ml (50 µM) DPPH solution made in methanol was added to each 2 ml of standard ascorbic acid solutions (10 to 50 µg/ml) and plant extract solutions (100 to 1000 µg/ml). Immediately after the addition, all samples were incubated in a dark place for 30 minutes. A control sample was prepared in the same manner by adding methanol (2 ml) to DPPH (2 ml solution). By using a UV-visual spectrophotometer, the absorbance of each sample was measured at 517nm, and the estimation of inhibition (%) of all samples was calculated by using the equation; Inhibition (%) = (Ac- As/Ac) x 100 where, Ac - Absorbance of control and As - Absorbance of sample/standard.The graph was plotted between % inhibition Vs different concentrations of both plant extracts and ascorbic acid. From the graph the IC_{50} value was determined[21].

***In vitro* acetylcholinesterase inhibitory activity by Ellman's method**

A modified 96-well microplate assay based on Ellman's method was used to measure acetylcholinesterase (AChE) inhibitory activity The AChE enzyme hydrolyzes acetylthiocholine to form thiocholine which reacts with Ellman's reagent (DTNB) to form 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate, the latter can be detected at 412 nm. In a 96-well plate, 100 µL of DTNB (3mM), 20µL of 0.26 U/ml AChE (in 0.1% BSA), 40µL of buffer (Tris-HCl 50mM, pH 8.0), and 20 µL of each extract in various concentrations (20-500µg/ml), were added to the wells in triplicate. After mixing, the plate was incubated for 15 min at 25°C. The enzyme reaction was started after the addition of 20 µL acetylthiocholine iodide (ATCI) 15 mM, and the hydrolysis of acetylthiocholine was monitored at 412nm every 5 minutes for the next 20 mins in a microplate reader, and the percentage inhibition was calculated [22].

% AChE inhibition = [(Ac -As)]/(Ac) × 100 (Ac = absorbance of the control(without sample); As = absorbance of the sample solutions;). IC_{50} values were obtained from the graph plotted between % AChE inhibition Vs extract concentrations.

Statistical analysis

All experiments were performed in triplicate and results are expressed as mean ± standard deviation. IC_{50} values were calculated by linear regression analysis of percentage inhibition against concentration using Microsoft Excel.

RESULTS AND DISCUSSION

Physicochemical parameters and preliminary phytochemical study

The outcome of physicochemical parameters of *M. jalapa* root powder are shown in Table 1. The total ash value was 10.12 ± 0.24%, acid-insoluble ash value was 0.52 ± 0.048% and water-soluble ash value was 3.5 ± 0.62%. The total ash value (10.12%) is the total amount of material left after ignition, including physiological minerals and adventitious (surface) inorganic matter. However, the low acid-insoluble ash (0.52%) indicates the absence of siliceous or earthen materials such as sand or soil[23]. The water-soluble ash value (3.5%) is the portion of total ash that is soluble in water and is mainly composed of water-soluble inorganic salts taken up by the plant from the soil. The moisture content (7.58±0.35%) is within the range generally considered acceptable for the microbial and chemical stability of powdered plant material during storage, as excessive moisture

(usually above 10–12%) is often linked with risk of microbial proliferation and hydrolytic degradation of phytoconstituents[24]. The water-soluble extractive value (16.52 ± 0.22 %) was higher than the alcohol-soluble extractive value (10.78 ± 0.16 %), which is consistent with the higher ability of water to dissolve polar bioactive constituents and non-bioactive polar bulk material such as sugars and mucilage present in the tuberous root. The water-soluble extractive value here (16.52%) is comparable with the value reported by Hanani et al. (2017) for *M. jalapa* (18.63%), and the alcohol-soluble extractive value (10.78%) is very close to their reported value of 11.02%[23]. The sequential root extracts were subjected to preliminary qualitative phytochemical screening. Our findings revealed alkaloids, phenolics, triterpenes, and steroids were present in all three extracts. The flavonoids and saponins were detected in the ethyl acetate extract and methanolic extract. Furthermore, the carbohydrates and proteins were detected only in the methanolic extract. (Table 2). These secondary metabolites are essential for many biological activities.

Table 1. Physicochemical parameters of *Mirabilis jalapa* root powder

Parameter	Value (% w/w)
Total ash value	10.12 ± 0.24
Acid-insoluble ash	0.52 ± 0.048
Water-soluble ash	3.5 ± 0.62
Moisture content	7.58 ± 0.35
Alcohol-soluble extractive	10.78 ± 0.16
Water-soluble extractive	16.52 ± 0.22

Table 2.Preliminary phytochemical study of chloroform, ethyl acetate and methanolic root extracts of *Mirabilis jalapa*

Phytochemical test	Chloroform Extract	Ethyl Acetate Extract	Methanolic Extract
Alkaloid	+	+	+
Flavonoid	-	+	+
Phenolics	+	+	+
Saponin	-	+	+
Carbohydrates	-	-	+
Protein	-	-	+
Triterpene	+	+	+
Steroids	+	+	+

+ = present, - = absent

Total phenolic, flavonoid, and alkaloid content

Total phenolic, flavonoid, and alkaloid contents of the chloroform, ethyl acetate, and methanolic root extracts are presented in Table 3. The TPC and TFC were calculated from linear regression equation of standard curves ($y = 0.005x + 0.194$, $R^2 = 0.999$ and $y = 0.011x - 0.009$, $R^2 = 0.999$). The TPC and TFC values increased with increasing solvent polarity, where the methanolic extract had the highest values (TPC = 175.8 ± 1.52 mg GAE/g; TFC = 35.18 ± 0.48 mg QE/g) followed by the ethyl acetate extract (TPC = 142.20 ± 1.12 mg GAE/g; TFC = 11.82 ± 0.64 mg QE/g) and the chloroform extract (TPC = 91.4 ± 1.25 mg GAE/g; TFC = 2.21 ± 0.52 mg QE/g). The progressive increase in TPC and TFC with increasing solvent polarity (methanol > ethyl acetate > chloroform) is consistent with the generally higher affinity of polar solvents for phenolic and flavonoid constituents. This similar pattern has been observed in earlier solvent-comparison studies using extracts of *M. jalapa* tuber where polar solvents (methanol, water) were more effective in extracting phenolic and flavonoid content than non-polar and mid-polarity solvents[25].

The total alkaloid content was also found to vary among the extracts. The methanolic extract showed the highest TAC **(**0.47 ± 0.026 mg AE/g**)**, followed by the ethyl acetate extract **(**0.33 ± 0.042 mg AE/g**)** and the chloroform extract **(**0.16 ± 0.034 mg AE/g**)**. The TAC was calculated from linear regression equation using the atropine calibration curve, ($y = 0.009x + 0.004$, $R^2 = 0.999$). The higher TAC in the methanolic extract may be associated with the extraction of relatively polar or protonatable alkaloidal constituents. However, alkaloids comprise a chemically diverse group of compounds with considerable variation in polarity, ionization state, and solvent affinity. Therefore, the distribution of total alkaloids among solvent fractions cannot be explained solely on the basis of solvent polarity. Further fractionation and structural characterisation (e.g. LC-MS/MS, NMR) would be needed to confirm the particular classes of alkaloids responsible for this observation.

Table 3. Total phenolic (TPC), flavonoid (TFC), and alkaloid (TAC) content of chloroform, ethyl acetate, and methanolic root extracts of *M. jalapa*

Extract	TPC (mg GAE/g)	TFC (mg QE/g)	TAC (mg AE/g)
Chloroform extract	91.4 ± 1.25	2.21 ± 0.52	0.16 ± 0.034
Ethyl acetate extract	142.20 ± 1.12	11.82 ± 0.64	0.33 ± 0.042
Methanolic extract	175.8 ± 1.52	35.18 ± 0.48	0.47 ± 0.026

DPPH radical scavenging activity and Acetylcholinesterase inhibitory activity

The DPPH radical scavenging activity of the three extracts, along with the ascorbic acid reference standard, is shown in Table 4. The percentage inhibition of all samples increased in a concentration-dependent manner. The methanolic extract showed the highest antioxidant activity among the three extracts (IC_{50} = 276.87 ± 0.39 µg/mL) followed by the ethyl acetate extract (IC_{50} = 449.05 ± 0.42 µg/mL) and the chloroform extract (IC_{50} = 880.81 ± 0.54 µg/mL). All extracts showed considerably weaker activity than the ascorbic acid standard (IC_{50} = 26.48 ± 0.16 µg/mL). The acetylcholinesterase inhibitory activity of the three extracts is shown in Table 5. The methanolic extract showed the highest inhibitory activity (IC_{50} = 76.99 ± 0.36 µg/mL) and was significantly more potent than the ethyl acetate extract (IC_{50} = 392.85 ± 0.32 µg/mL) and the chloroform extract (IC_{50} = 491.88 ± 0.58 µg/mL).

Table 4. DPPH radical scavenging activity of chloroform, ethyl acetate, and methanolic root extracts of *M. jalapa*

Concentration (µg/mL)	Ascorbic acid (%inhibition)	Concentration (µg/mL)	%inhibition		Concentration (µg/mL)	Chloroform Extract (%inhibition)
			Methanolic Extract	Ethyl acetate Extract		
10	20.62 ± 0.16	100	20.34 ± 0.11	16.32 ± 0.26	200	22.99 ± 0.32
20	39.48 ± 0.22	200	40.53 ± 0.27	29.19 ± 0.42	400	33.78 ± 0.21
30	56.67 ± 0.10	300	55.25 ± 0.35	36.39 ± 0.36	600	42.01 ± 0.44
40	73.89 ± 0.18	400	69.64 ± 0.44	43.59 ± 0.20	800	46.03 ± 0.16
50	89.78 ± 0.24	500	82.46 ± 0.30	55.83 ± 0.21	1000	54.16 ± 0.22
IC_{50} (µg/mL)	**26.48 ± 0.16**	**IC_{50} (µg/mL)**	**276.87 ± 0.39**	**449.05 ± 0.42**	**IC_{50} (µg/mL)**	**880.81 ± 0.54**

Table 5. Acetylcholinesterase inhibitory activity of chloroform, ethyl acetate, and methanolic root extracts of *M. jalapa*

Concentration (µg/mL)	%inhibition		Concentration (µg/mL)	Methanolic Extract (%inhibition)
	Ethyl acetate extract	Chloroform Extract		
100	19.34 ± 0.20	18.77 ± 0.42	20	10.3 ± 0.12
200	30.76 ± 0.41	24.45 ± 0.36	40	20.61 ± 0.33
300	39.12 ± 0.22	31.72 ± 0.18	60	35.56 ± 0.40
400	53.24 ± 0.56	43.67 ± 0.52	80	55.0 ± 0.24
500	60.52 ± 0.28	51.34 ± 0.30	100	66.49 ± 0.52
IC_{50} (µg/mL)	**392.85 ± 0.32**	**491.88 ± 0.58**	**IC_{50} (µg/mL)**	**76.99 ± 0.36**

The antioxidant and acetylcholinesterase inhibitory activities exhibited a similar pattern, with the methanolic extract demonstrating the highest activity, followed by the ethyl acetate and chloroform extracts. This pattern was consistent with the observed TPC, and TFC values, suggesting that phenolic and flavonoid constituents may contribute to the antioxidant and acetylcholinesterase inhibitory activities of the root extracts. These findings are consistent with previous reports suggesting that phenolic and flavonoid constituents may contribute to antioxidant and acetylcholinesterase inhibitory activities in medicinal plants, including different parts of *M. jalapa* [25-30].

Overall, the findings indicate that the methanolic extract was the most promising fraction among the extracts investigated**,** exhibiting the highest TPC, TFC, and TAC together with the strongest DPPH radical-scavenging and acetylcholinesterase inhibitory activities. These findings support the potential of *M. jalapa* root methanolic extract as a source of bioactive phytoconstituents with antioxidant and acetylcholinesterase inhibitory potential.

CONCLUSION

This study provides a comparative, solvent-resolved assessment of the phytochemical composition, antioxidant and acetylcholinesterase inhibitory activities of *Mirabilis jalapa* root extracts. Among the three extracts evaluated, the methanolic extract exhibited the highest phenolic, flavonoid and alkaloid contents and the strongest antioxidant and acetylcholinesterase inhibitory activities, indicating that methanol was the most effective solvent for extracting these bioactive constituents from the roots. These findings support further investigation of *M. jalapa* roots, particularly their polar extracts, as potential sources of natural antioxidant and acetylcholinesterase inhibitory compounds with potential relevance to oxidative stress and cholinergic dysfunction.

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