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Antioxidant and Anticancer Activity of *Rhynchosia rufescens* (Willd.) DC.

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The present study deals with the chemical composition, antioxidant, antimicrobial and anticancer efficacy of *Rhynchosia rufescens* (RR) leaf methanol (RRME), ethyl acetate (RREAE) and petroleum ether (RRPE) extracts. Preliminary phytochemical results revealed that alkaloids, coumarins, flavonoids, volatile oils, fatty acids, and phenols were present in the test extracts. Gas chromatographic analysis of RRPE revealed the identification of 25 volatile compounds. The oil contains monoterpenes (9.6%) and fatty acids (6.12%). The major components of the oil are beta- copaene (32.06%), cyclofenchene (16.53%), beta- pinene (5.69%), 3-carene (3.48%) and sabinene (3.22%). Quantitative estimation of total phenols (TPC) and flavonoids (TFC) indicated that TPC ranged from 8.99 ±1.00 to 60.00±1.15 mg GAE/g dry weight and TFC was between 4.98±0.08 to 20.4±0.50 mg QE/ g dry weight respectively. Methanol extract showed the highest phosphomolybdenum dependent antioxidant capacity, while ethyl acetate extracts strongly inhibited DPPH and hydroxyl radicals. Anticancer study results revealed that ethyl acetate extract enhanced caspase-3 mediated DNA fragmentation in HCT – 116 cell lines than standard cisplatin. In conclusion, *R. rufescens* extracts showed potent antioxidant, antibacterial activity and potential anticancer activity in HCT-116 cell lines. The observations of this study indicated that *R. rufescens* extracts are a rich source of natural antioxidants and anticancer components.

Keywords: Antibacterial, Essential oil composition, Flavonoid, HCT-116 cell lines, Phenolic

An uncontrolled division of cells leads to cancer development. There are about 277 types of cancers, of which colon/colorectal cancer causes mortality of about 9.4% (Hyuna Sung *et al*., 2021). Colon cancer is the second cause of death worldwide (Sanower Hossain *et al*., 2022). The drugs used in chemotherapy to treat colon cancer cause side effects such as fatigue, hair loss, vomiting, diarrhea, nerve damage, *etc*. Demand for drugs with potent anticancer activity with minimal side effects has been increasing day by day. Medicinal plants used in traditional medicine were found to have a rich source of phyto-constituents with potential therapeutic value. According to WHO estimations, nearly 80% of the World's population depends on herbal medicine for their primary need. Natural products isolated from plants have been shown to have therapeutic potential against cancer and other inflammatory diseases (Malsawmdawngliana *et al*., 2021; Nghakliana *et al*., 2021; Jenifer *et al*., 2021; Kaan, 2022; Sathelly *et al*., 2023 and Mobasher *et al*., 2023).

Leguminosae, or Fabaceae, is the third largest family after Orchidaceae and Asteraceae. *Rhynchosia* is one of the important genera of this family, commonly known as wild pulses, and consists of about 300 species distributed in tropical and sub-tropical areas worldwide. India is represented by 22 species and 13 are present in Andhra Pradesh (Chadha, 1976; Willis, 1966; Pullaiah *et al*., 1997). Previous phytochemical reports on the genus revealed that the species are rich in flavones, flavone glycosides, flavanones, dihydro-flavonols, simple phenolics, sterols (Rammohan *et al*., 2020) and essential oils (Bakshu and Venkata Raju, 2001 and 2009). Chemical constituents isolated from different *Rhynchosia* species and their extracts reported various pharmacological properties like antioxidant, antimicrobial, anticancer and anti-inflammatory (Rammohan *et al*., 2015; Praveena *et al*., 2013; Jia *et al*., 2015).

Rhynchosia rufescens (Willd.) DC. is a slender pubescent twining shrubs. Leaves elliptic-ovate with a characteristic aromatic odor. The flowers are yellow with maroon streaks. The species is common in dry deciduous forests of the Eastern Ghats of Andhra Pradesh (Venkata *et al*., 1995). In folk medicine, leaves are reported as a potential abortifacient and used to treat skin diseases by the Adivasis inhabiting forests of Andhra Pradesh and the mannar tribes of Kerala

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(Vishnu, 2021; Ajesh *et al*., 2012). Phytochemical constituents isolated from *R. rufescens* leaves were vitexin, isovitexin, Vicenin-2, Isoorientin, Lucenin-2 (Adinarayana *et al*., 1979), Orientin (Ali *et al*., 1992), Kaempferol-3-O-methylether, Quercetin-3-O-methylether (Adinarayana & Ramachandraiah, 1984). The present study aimed to evaluate the chemical composition of essential oil, quantitative estimation of phenolic and flavonoid contents, antioxidant, antibacterial and anticancer effects of *R. rufescens* leaves organic extracts.

Materials and Methods

Plant material

Rhynchosia rufescens (Willd.) DC. material was collected from Nallamala forest, Eastern Ghats of Andhra Pradesh (Fig. 1). The collected specimens were authenticated by using local and regional floras (Venkata *et al*., 1995; Gamble, 1935). The voucher specimen (#298) was deposited in the Botany Department, Rayalaseema University, Andhra Pradesh, India.

Preparation of Extracts

The collected leaves were cleaned manually, rinsed with tap water, and dried under shade. The leaves are powdered using a mixer grinder and stored in airtight containers at 4°C. One hundred grams of plant material was loaded onto Soxhlet and successively extracted with petroleum ether (RRPE), ethyl acetate (RREAE), and methanol (RRME). The solvent was separated from extracts using a distilled unit. The unprocessed extracts were subjected to phytochemical, antioxidant and anticancer assays using *in vitro* methods.

Phytochemical analysis

Preliminary phytochemical screening

The concentrated RRPE, RREAE and RRME extracts obtained from *R. rufescens* were subjected to qualitative phytochemical analysis using standard methods (Das and Bhattacharjee, 1970; Gibbs, 1974; Amarasingham *et al*., 1964 and Harborne, 1991) to know the distribution of different group of secondary metabolites.

Fig. 1 — *Rhynchosia rufescens* Flowering Condition

Estimation of total phenolic content (TPC)

The TPC of RRPE, RREAE and RRME extracts was quantified by the standard FCA reagent method (Singleton & Rossi, 1965). The extracts were mixed with 100 µL of FCA reagent and allowed 5 - 8 min for incubation at room temperature. Then 0.58 mL of distilled water was added to the solution along with 300 µL of Na2CO3 (20%) solution to make up the total volume of 2 mL. The reaction mixture was incubated for 2 h. The intensity of the color reaction has been measured in terms of optical density (OD) which was read at 765 nm. The experiment is appropriately compared and standardized with the blank and positive control (Gallic acid) using a spectrophotometer.

Estimation of total flavonoid content (TFC)

The TFC of RRPE, RREAE and RRME extracts were determined by using the standardized method (LakshmanRaju, 2012). The reaction mixture consisting of aliquots (50 and 100 µL) of the extracts and the sodium nitrite (5%) aluminum chloride (10%) was incubated for 5 min at 25°C. Then, the aliquots were neutralized by treating with 1 M sodium hydroxide and allowed the reaction for 15 min at 25°C. The optical density (OD) values of the resultant solutions were measured at λ 510 nm.

Gas Chromatography and Mass Spectroscopic Studies of RRPE

Gas chromatographic analysis of RRPE was carried out by using the methods mentioned (Venkata Ratnam *et al*., 2010). The components were identified by cross-checking the RI values reported in the literature (Adams, 2001).

Antioxidant Studies

Ammonium molybdate dependent antioxidant activity

Ten microliters of RRPE, RREAE and RRME were treated with 1mL of phosphor molybdenum reagent. Then, the samples were kept in the dry bath at 95 ºC for 1 ½ h. The O.D. values of each sample was measured at 695λ against blank in the UV-Vis spectrophotometer (Umamaheshwari & Chatterjee, 2008). The antioxidant capacity of each extract was expressed as milligrams (mg) of ascorbic acid equivalent per gram dry weight of the extract.

DPPH reducing activity

Different concentrations of RRPE, RREAE and RRME extracts were mixed with one mL of methanolic-DPPH solution (4 mg/L). The solutions were incubated for 30 min in the dark chamber at ambient temperature. After completion of the time, the DPPH solution and test samples' absorbance were recorded at 517 λ against the blank in the UV-spectrophotometer (Braca *et al*., 2002).

Hydroxyl radical scavenging assay

The hydroxyl radical inhibitory activity of the RRPE, RREAE and RRME were determined by the ferric chloride – EDTA- ascorbic acid and H2O2 system based method (Halliwell *et al*., 1987). Briefly, different concentrations of the RRPE, RREAE and RRME extracts were mixed with 2-deoxy-ribose (10 mM), ascorbic acid (1 mM), EDTA (1 mM), and H2O2 (10 mM), then the solutions were kept at 37°C for 1 h. After that, 0.5% trichloro acetic acid, 25 mMNaOH and TCA (10%) were added to each test tube and allowed the reaction at 100°C for 20 min. After attaining the ambient temperature, the O.D. values of each sample was taken at 532 nm against the control. The positive effect of the result has been compared to the standard natural antioxidant, *i.e*., Ascorbic acid.

Antibacterial studies

Microorganisms used

The microorganisms used in the present study are *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* to test the extracts. The organisms were purchased from the MTCC center, IMTECH, Chandigarh, India.

Antimicrobial activity

The antibacterial efficacy of RRPE, RREAE and RRME was studied using the disc diffusion method (Cruikshank, 1975). Paper discs impregnated with distinct concentrations of plant extracts were placed on the petri plates, containing 20 mL of nutrient agar media seeded with 0.1 mL of overnight grown microbial suspension. The discs saturated with methanol, ethyl acetate, and petroleum ether were negative controls. Bacterial free zones present around the discs treated as positive results. The zones were measured after 24 h and tabulated.

The minimum inhibitory concentration (MIC)

The MIC of each extract against tested pathogens was assessed using the broth dilution method (NCCLS, 1999). Different concentrations of the extracts, starting from 2 mg to 125 mg/mL, were tested in this method. 100 µL of the microbial suspension was added to each tube. Tubes were kept in an incubator at 37°C for 18 h and then OD. values were measured with colorimetry at 620 nm. The extract showing no visible growth was considered as its MIC value. DMSO served as the negative control.

Cell culture studies

Cell culture

Human Colorectal adenocarcinoma cell line (HCT-116) purchased from NCCS, Pune. The cells were cultured in DMEM- High Glucose medium containing fetal bovine serum in a CO_2 incubator.

MTT Cell viability study

HCT-116 cell **(**200µL) suspension was seeded in a 96 well plate at 20,000 cells per well, without the test sample. Cells were allowed to grow for 24 h, then treated with RRPE, RREAE, RRME, at 25, 50, 100, 200 and 400 ug/mL concentrations and standard drug as control.

The plates were incubated for 24 hrs at 37°C in a 5% CO2 atmosphere. After that, the plates were removed from the incubator, separated spent media and added MTT reagent (0.5 mg/mL). Further, the cells were incubated at 37°C for 3 h. After that, the MTT reagent was removed and added 100 µL of solubilization solution (DMSO). The plates were gently stirred in a shaker to enhance dissolution. After that, the OD values of each sample were measured using an ELISA reader at 570 nm and 630 nm. Cell viability was calculated according to standard methods (Lombardi *et al*., 2017; Alley *et al*., 1986).

Measurement of caspase-3 activity by ELISA

The cells were cultured (5.3×10^4 cells) in 2 mL of culture medium in six-well plates. The cells were treated with a predetermined IC_{50} value of the RRPE, RREAE and RRME for 24 h; the attached cells were rinsed with cold PBS, then the cells were suspended in PBS and subjected to one freeze//thaw cycle at room temperature. The lysed cells were centrifuged at 2000 rpm for 10 min. The supernatant was collected and stored at -80°C. The supernatant was subjected to ELISA to quantify caspase-3 activity (RayBio® Human Caspase-3 ELISA Kit).

Determination of DNA fragmentation by TUNEL assay using Flow Cytometry analysis

The effect of RRPE, RREAE and RRME on DNA fragmentation was assessed using TUNEL assay.

Cells (1-2 × 10^6/well) were cultured in six-well plates with or without *R. rufescens* extracts at 0, 25, 50, 75, or 100 µg/mL for 24 h. After treatment, cells were subjected to determine DNA fragmentation following previous reports (Enari *et al*., 1998; Li *et al*., 1995). The quantity of DNA breakup in selected cells treated with RRPE, RREAE, RRME and cisplatin was quantified by Flow cytometer (BD FACS Calibur).

Statistical analysis

The total phenol content (TPC) of water extracts was estimated using the gallic acid standard curve. The quantity has been expressed in milligrams of gallic acid equivalents per gram dry weight (mg GAE/g dwt.). The TFC was estimated using Quercetin as the standard component and was represented in milligrams of Quercetin present per gram dry weight of the extract (mg QE/g dwt.). Ascorbic acid (Vitamin C) was used as a standard and positive control and the antioxidant capacity (TAC) of the test extracts were calculated from the standard curve; the results were represented as mg of ascorbic acid equivalents per gram dry weight (mg ASE/g dwt.). The percentage inhibition of DPPH and hydroxyl radical scavenging capacity of selected medicinal plant water extracts was calculated using the following formula.

$$\% \text{ Inhibition of free radical} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100$$

Results

Yield of the extracts

R. rufescens leaves collected from the forests of the Eastern Ghats of Andhra Pradesh and successfully extracted with petroleum ether, ethyl acetate, and methanol. Petroleum ether extract (RRPE) yielded a greenish yellow color oily extract with aromatic odor (1.8%), ethyl acetate (RREAE) extract yielded a green color extract (3.2%) and methanol (RRME) yielded a greenish brown color extract (5%), respectively.

Table 1 — Preliminary phytochemical analaysisof *Rhynchosia rufescens* Extracts

Chemical test	RRPE	RREAE	RRME
Alkaloids	+	+	+
Coumarins	+	+	+
Flavonoids	+	+	+
Phenols	+	+	+
Terpenoids	-	+	+
Tannins	-	+	+
Volatile oils	++	-	-
Fixed oils	+	-	-

Phytochemistry

Qualitative phytochemical studies

Preliminary phytochemical analysis of RRPE, RREAE and RRME extracts was carried out using standard methods and revealed that alkaloids, coumarins, flavonoids and phenols were present in the test three extracts. Volatile oils and fatty acids were noticed in RRPE, while terpenoids and tannins were present in RREAE and RRME (Table 1).

Total phenolic and flavonoid content

The phenolic content of the leaf extracts of *R. rufescens* was determined using the standard curve of gallic acid and showed the range of 8.99 ±1.00 to 60.00±1.15 mg GAE/g dwt., while TFC was quantified using the standard linear graph of Quercetin. The TFC of the *R. rufescens* leaf extracts varied from 4.98±0.08 to 20.4±0.50 mg QE/ g dry weight. (Fig. 2).

GC-MS Analysis of RRPE

R. rufescens leaf petroleum ether extract yielded greenish yellow color oil (1.8%) with a characteristic odor. GC-MS analysis of the oil resulted in the identification of 25 components (Fig. 3). The chemical composition of the greenish-yellow oil is depicted (Table 2). The oil contains monoterpenes (9.6%) and fatty acids (6.12%). The major components of the oil are beta- copaene (32.06%), cyclofenchene (16.53%), beta- pinene (5.69%), 3-carene (3.48%) and sabinene (3.22%) (Table 2).

Antioxidant Studies

The total antioxidant capacity of *R. rufescens* leaf extracts (Fig. 4) revealed that methanolic extract showed a higher amount of antioxidant potential (90.00±1.54 mg ASE/g dwt.), followed by that of ethyl acetate (60.00±9.12 mg ASE/g dwt.) and petroleum ether (25.90±1.03 mg ASE/g dwt.).

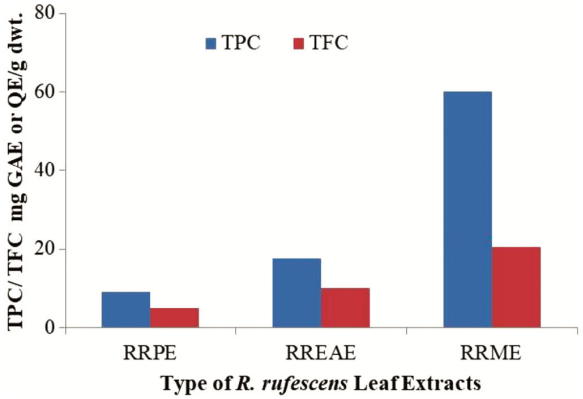

Fig. 2 — Total phenolic and flavonoid content of *R. rufescens* leaves extracts

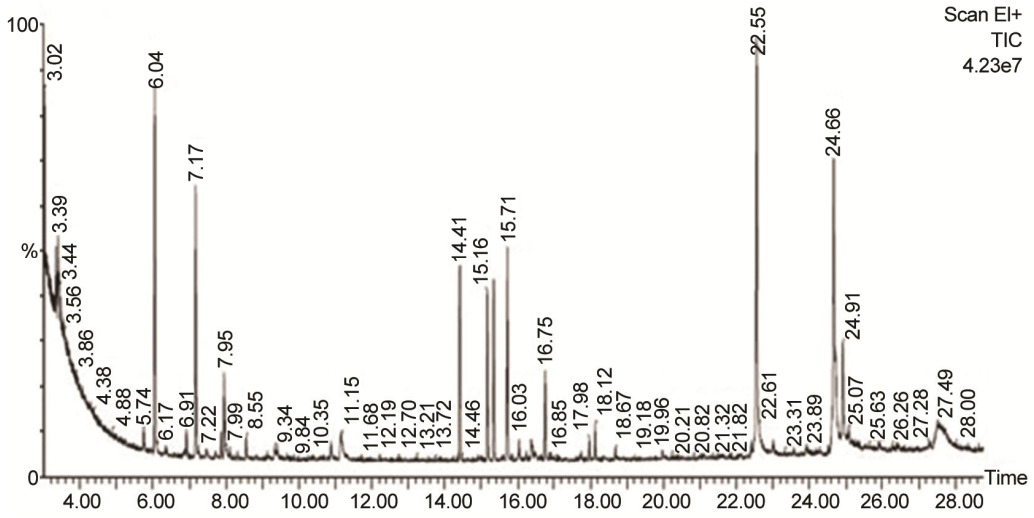

Fig. 3 — Gas Chromatogram of *R. rufescens* leaves petroleum ether extract

Table 2 — Chemical composition of *R. rufescens* Petroleum ether extract

Peak No.	RT Value	Compound Name	% of the Compound
1	3.018	Cyclofenchene	16.532
2	3.334	3-Carene	3.485
3	3.394	β-Pinene	3.91
4	4.66	Terpenen-4-ol	1.202
5	4.884	Verbenone	1.482
6	5.104	º-Pinene	0.280
7	5.229	Camphene	1.227
8	5.739	O0Cymene	0.225
9	6.040	Sabenene	3.222
10	6.915	à-Phellandrene	0.232
11	7.165	à-myrcene	2.159
12	7.945	D-Limonene	0.817
13	11.162	α-Terpineol	0.668
14	14.408	à-Cububene	1.667
15	15.163	Caryophyllene	1.390
16	15.333	à-Farnesene	1.504
17	15.708	Humulene	1.936
18	16.029	à-Copaene	0.242
19	16.749	à-Cubebene	0.860
20	18.119	4,6 Decadiene	0.273
21	22.546	α-Copaene	32.061
22	24.662	Myristoleic acid	4.964
23	24.907	Octadecanoic acid	1.160
24	27.558	Cyclohexanone, 2-(2nitro-1-phenyl-2-propenyl)-, (R*, R*)-	1.592
25	27.708	2,4,-Dichloro-4-methoxydiphenyl ether	0.495

DPPH quenching activity

DPPH is the most commonly used method to test the antioxidant activity of medicinal plant extracts or isolated compounds. All the test extracts reduced DPPH purple color in concentration dependent manner (Table 3). Ethyl acetate extract strongly inhibited DPPH radical, IC50 value (250 µg/mL).

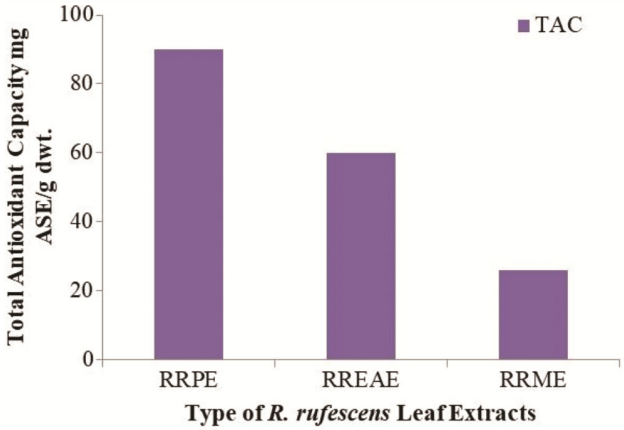

Fig. 4 — Total antioxidant capacity of *R. rufescens* leaves extracts

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging capacity of *R. rufescens* extracts was depicted (Table 4) and are dependent on concentration. Ethyl acetate extract strongly inhibited hydroxyl radicals, 50% at 15 µg/mL.

Antibacterial Studies

Antibacterial activity of RRPE, RREAE and RRME leaf extracts was determined by disc diffusion method using *in vitro* methods. The antibacterial activity against the test pathogens revealed that *P. aeruginosa* was strongly affected by RRME (18.66±2.61 mm) at 25mg/mL. While *B. cereus* was significantly inhibited by RRPE (18.66±0.57 mm) at 25 mg/mL and RREAE extract exhibited moderate activity against the test pathogens at 25 mg/mL (Table 5).

The Minimum Inhibition Concentration (MIC) values of the *R. rufescens* leaf extracts were determined by the

Table 3 — DPPH Scavenging activity of *R. rufescens* leaf extracts

RRPE		RREAE		RRME		Gallic acid	
Con. mg/mL	% inhibition	Con. mg/mL	% inhibition	Con. mg/mL	% inhibition	Con. µg/mL	% inhibition
0.1	10.82±0.2	0.1	20.25±1.48	0.2	11.25±1.48	1	20.10±1.10
0.2	34.02±0.4	0.2	30.15±2.19	0.3	24.15±2.19	2	33.21±0.21
0.3	38.14±2.91	0.25	50.11±1.42	0.4	39.11±1.42	4	52.24±0.33
0.4	50.81±5.47	0.3	60.26±1.2	0.45	51.26±1.2	6	60.14±0.12
0.5	68.12±1.12	0.4	75.05±2.89	0.5	70.05±2.89	8	72.23±1.22
0.6	81.96±2.18	0.5	83.15±1.48	0.6	73.15±1.48	10	80.41±2.11
0.7	90.1±2.19	0.6	98.2±1.18	0.7	81.2±1.18	12	91.24±2.13
						14	92.23±1.33

Table 4 — Hydroxyl radical scavenging activity of *R. rufescens* leaf extracts

RRPE (µg/mL)	% inhibition	RREAE (µg/mL)	% inhibition	RRME (µg/mL)	% inhibition	Gallic acid (µg/mL)	% inhibition
50	22.12±0.12	10	26.22±1.12	20	22.12±1.01	5	08.01±0.4
80	49.9±1.14	15	50.11±1.14	30	51.24±2.11	10	24.01±0.2
100	50.01±0.21	20	62.22±2.14	60	55.34±1.11	15	39.21±1.1
150	60.12±1.12	30	74.31±2.22	80	61.72±0.11	20	50.11±0.21
200	62.24±0.44	40	82.12±1.12	90	66.81±1.01	25	60.04±0.1
250	68.33±0.55	50	98.12±0.22	100	75.12±2.19	30	74.00±1.02
300	70.22±0.66			120	82.15±2.01	35	80.22±1.21
350	73.15±0.77			150	95.16±1.14	40	92.01±2.01

Table 5 — Antibacterial activity of RRPE, RREAE and RRME extracts

Organisms	Zone of inhibition mm^{-1}									
	RRPE			RREAE			RRME			
	mg/mL		MIC µg/mL	mg/mL		MIC µg/mL	mg/mL		MIC µg/mL	Standards µg/mL
	10	25		10	25		10	25		
Bacillus cereus MTCC1429	11.66±0.57	18.33±0.57	156±0.0	8.330.57	9.33±0.57	625±1.22	9.66±0.57	11.66±0.57	156±0.0	18.66±1.16
Staphylococcus aureus MTCC737	12±1.0	16.66±1.15	312±0.15	9.330.57	10.33±0.57	625±1.22	7.66±0.57	10.66±1.15	156±0.0	18.66±1.16
Escherichia coli MTCC1687	10.33±0.57	13.66±0.57	312±0.15	10.330.57	12.33±2.51	312±0.15	9.0±1.0	12.00±0.0	156±0.0	36.33±1.52
Pseudomonas aeruginosa MTCC1688	10.66±1.15	17.66±0.57	312±0.15	10.330.57	14.66±1.15	156	12±2.0	18.66±2.61	78.0±0.57	36.33±1.52

broth dilution method. Among the test extracts, water extract exhibited significant inhibition against the test pathogens like *P. aeruginosa*, which was significantly affected by RRME (MIC value 78 µg/mL) and RREAE (MIC value 156 µg/mL), while *B. cereus* was strongly inhibited by RRPE (MIC value 156 µg/mL) (Table 5).

Cell culture studies

Cytotoxic Studies

Cytotoxic effects of RRPE, RREAE and RRME were assessed against HCT-116 cell lines using *in vitro* studies. The extracts were tested at various concentrations, from 25 µg/mL to 400 µg/mL. All the tested extracts exhibited concentration dependent cytotoxic effects on HCT-116 cell lines, revealing that ethyl acetate extract strongly (94.81%) suppressed the proliferation of HCT-116 cell lines at 400 µg/mL, followed by petroleum ether (90.37%) and methanol (86.81%) (Fig. 5). Based on the potential cytotoxic effect of extracts against HCT-116 cells, further studies, *i.e.*, ELISA and TUNEL assays, are assessed with HCT-116 cells to know the anticancer activity of the extracts.

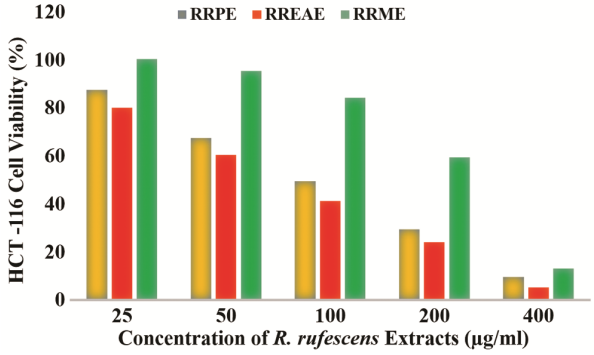

Fig. 5 — Cytotoxic effects of *R. rufescens* leaves extracts on colon cancer cell lines

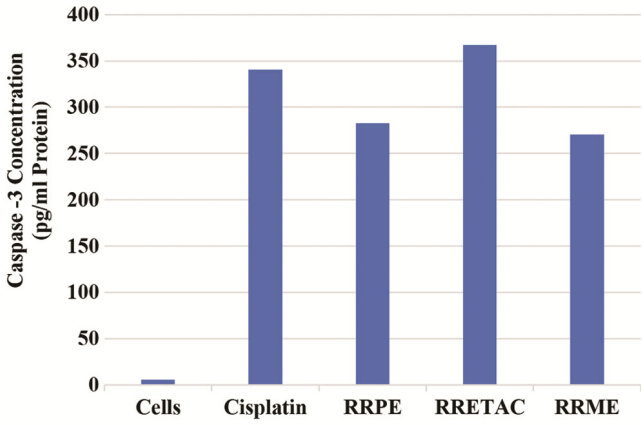

Fig. 6 — Effect of *R. rufescens* extracts on Caspase – 3 activities in HCT -116 cell lines

Quantification of caspase -3 by ELISA method

IC_{50} of the extracts was calculated from MTT assay and was subjected to caspase-3 activity in HCT 116 cell lines. Among the test extracts, RREAE exhibited the highest caspase -3 activity (367.56 pg/mL), followed by RRPE and RRME extracts (Fig. 6).

Effect of R. rufescens extracts on DNA fragmentation in HCT-116 cell lines

The effect of RRPE, RREAE and RRME on DNA fragmentation in HCT-116 cell lines was measured by TUNEL assay using flow cytometry analysis. The results revealed that the cells treated with ethyl acetate extract showed more DNA damage (96.68) than that of standard cisplatin (91.31), followed by petroleum ether and methanol extracts (Fig. 7A & B).

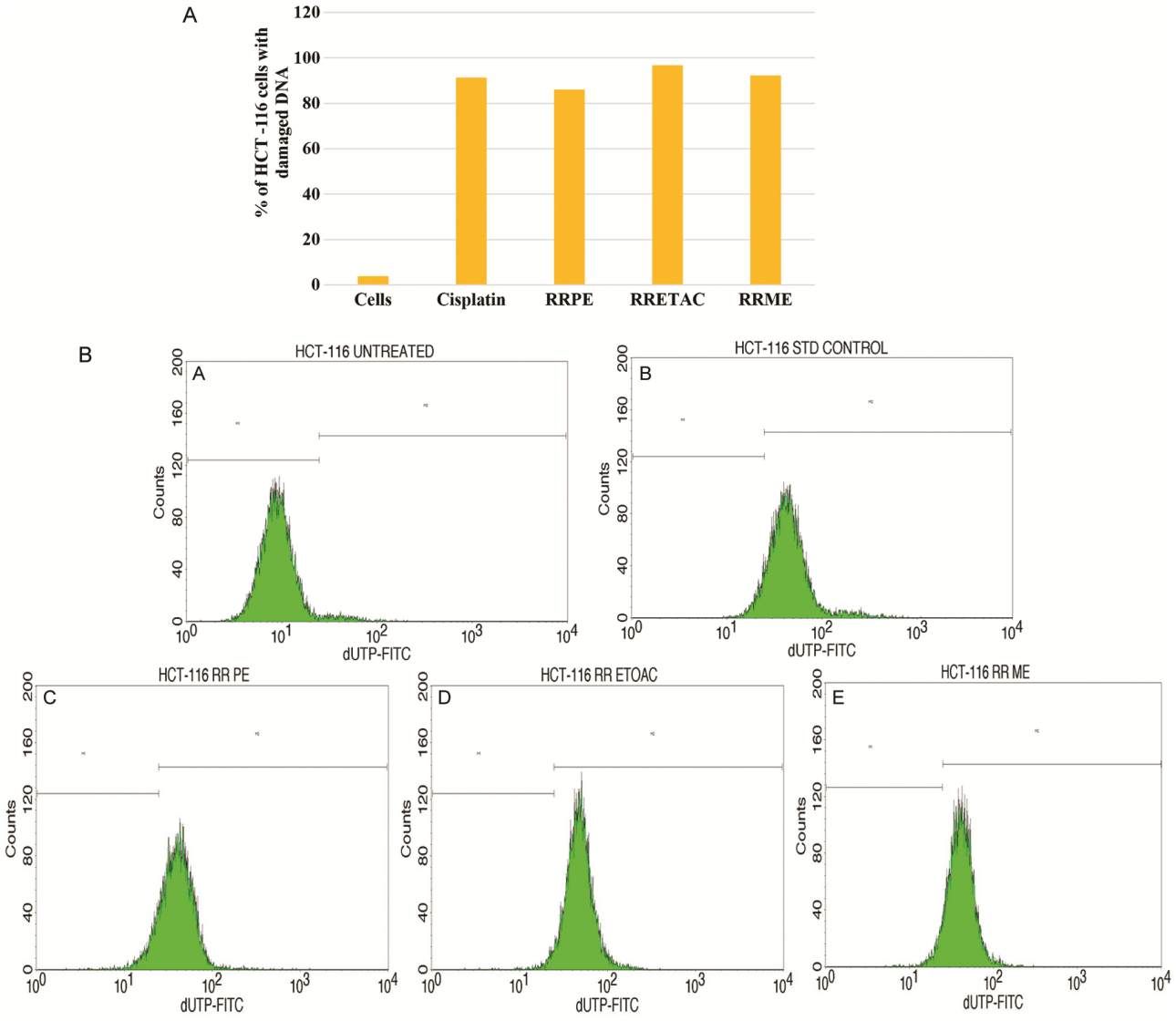

Fig. 7 — (A) Effect of *R. rufescens* extracts on DNA fragmentation in HCT -116 cell lines; and (B) Effect of *R. rufescens* extracts on DNA fragmentation in HCT -116 cell lines

Discussion

The plant-based drugs/ constituents are known as specific targets in chemotherapy. Commercially, only 140 approved anticancer drugs are available to treat more than 200 types of cancers. Apart from chemotherapy, other methods used to treat Cancer are radiotherapy, surgical therapy, immunotherapy and hormonal therapies, which are associated with side effects such as severe damage to healthy cells and alterations in metabolic, hormonal and immune systems (Shukla & Pal, 2004; Latosinska *et al*., 2013). Hence, there is a need to search for alternative and safe novel drugs of natural origin necessary to treat Cancer. Previous phytochemical reports on *Rhynchosia* species showed that 77 compounds were isolated, including flavonoids, isoflavonoids, xanthones, simple polyphenols and sterols (Rammohan *et al*., 2020). The present study results on qualitative phytochemical tests of *R. rufescens* revealed the presence of alkaloids, flavonoids, coumarins, phenols, tannins and oils. Quantitative phytochemical analysis of total phenolic and flavonoids of the extracts revealed that methanol extract extracted more phenolic and flavonoids content than petroleum ether and ethyl acetate. This may be due to the higher solubility of these compounds in methanol than the other solvents tested (Do *et al*., 2014). The highest phenolic and flavonoid content of the extracts was reported in *Severinia buxifolia* (Dieu-Hien Truong *et al*., 2019) and vegetables (Babbar *et al*., 2014).

Phytochemical constituents isolated from *R. rufescens* leaves are Orientin, Iso-orientin and Lucenin (Adinarayana & Ramachandraiah, 1984). Gas chromatography and mass spectral analysis of *R. rufescens* leaf essential oil revealed the presence of 25 components (Table 2). β-copane (32.06%), cyclofenchene (16.53%), β-pinene (5.69%), myristolic acid (4.96%) and β- careen (3.485) are the major constituents of the oil. Of the 25 components identified 8 components were reported in essential oils of *R. beddomei* (Bhakshu & Venkata Raju, 2001), *R. heynii* (Bhakshu & Venkata Raju, 2009) and *R. minima* (Gundedza *et al.*, 2009). Major components identified in the oil are β-copane (Turkez *et al*., 2014) and β-pinene (Salehi *et al*., 2019), reported for antioxidant and cytotoxic properties.

The antioxidant and antiradical activity of *R. rufescens* extracts was assessed using ammonium molybdate, DPPH and hydroxyl radical scavenging methods. The antioxidant effect of ethanolic extract of *R. beddomei* against DPPH, nitric oxide, H_2O_2 and superoxides was reported (Jaya *et al*., 2013). DPPH scavenging capacity of isolated active principles from *R. suaveolens, R. capitata* and *R. minima*, mangiferin, isoorientin, vitexin and polysaccharide fractions were studied (Aluru Rammohan *et al*., 2015; Praveena *et al*., 2013; Jia *et al*., 2015).

The antibacterial activity of RRPE, RREAE and RRME was studied by disc diffusion method. Among the test extracts, RRME exhibited the highest zone of inhibition on *Pseudomonas aeruginosa* (18.66±2.61) and RRPE exhibited highest zone of inhibition against *Bacillus cereus* (18.66±0.57), which is equal to that of standard drug ampicillin. The intense antibacterial activity of petroleum ether extract may be due to the presence of cyclofenchane, β-pinene, caryophyllene and β-copaene (Hazhedarogu *et al*., 2001; Rivas da Silva *et al.,* 2012 and Dahhan *et al.,* 2015), which were reported to possess antibacterial activity, strongly supported by the results, received during the present study.

Uncontrolled cell proliferation/ growth is a characteristic feature of Cancer. Induction of apoptosis and suppression of cancerous cell proliferation are assumed to be the best procedures for cancer therapy (Woo *et al*., 1998; Shi *et al*., 2002). Caspases represent the cysteine proteases family, which are responsible for initiating programmed cell death by inducing DNA fragmentation, cell shrinkage, and membrane blebbing. Among the caspases, caspase -3 plays a vital role in inducing apoptosis, and it is believed to be a biomarker for programmed cell death in cancer cells (Sui *et al*., 2016). In the present study, *R. rufescens* extracts strongly suppressed the growth of HCT116 cell lines (dose-dependent). Quantification of caspase -3 in cancerous cells is considered the best way for the determination of apoptosis. So, further, we quantified caspase-3 concentration in HCT-116 cell lines treated with *R. rufescens* petroleum ether, ethyl acetate and methanol extracts using ELISA method. The results showed that ethyl acetate extract strongly suppressed the growth of selected cancerous cells by caspase-3 activity (367.56 pg/mL) than petroleum ether and methanol extracts. The extract activity is more potent than standard cisplatin (340.35 pg/mL). Several scientific studies reported that medicinal plant extracts and nano-particles of herbal drugs (Biswas *et al*., 2022; Nghakliana *et al*., 2021 and Konduri *et al*., 2022) and bioactive phyto-constituents isolated from medicinal plants as potent anticancer or cytotoxic drugs

(Shafique M & Sarma, 2022; Priyanka Singh *et al*., 2023 and Priyadarsini *et al*., 2023).

To confirm the potential caspase activity of *R. rufescens* petroleum ether, ethyl acetate and methanol extracts, subjected the extracts to TUNEL assay using flow cytometry analysis to detect the intracellular changes (DNA fragmentation) in colon cancer cell lines. FACS analysis results showed that the extracts at tested concentrations, *i.e*., PE (94.63 µg/mL), ethyl acetate (75.72 µg/mL) and methanol (234.82 µg/mL), exhibited DNA fragmentation in HCT-116 cell lines. Among the test extracts, the cells treated with ethyl acetate extract exhibited more DNA fragmentation (96.68%) than control cells and other extracts. The extract is more potent than the standard cisplatin (91.31%).

Conclusion

The present study concludes that *R. rufescens* RRPE, RREAE, and RRME are rich in volatile components, phenolics, and flavonoids. The extracts showed a good amount of phosphomolybdenum dependent antioxidant and strong DPPH and hydroxyl radical scavenging activities. Further, the extracts strongly suppressed the growth of the colon cancer cell lines. RREAE strongly enhanced caspase-3 activity and DNA fragmentation in HCT116 cell lines. The research observations of the present study suggest that *R. rufescens* could be used as a potential source of natural antioxidant/antibacterial and anticancer components. Further molecular studies are required to isolate and characterize active principles and to validate their pharmacological properties in *in vivo* models.

Conflict of interest

All authors declare no conflicts of interest.

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