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Exploration of *in vitro* Antioxidant and Cytotoxic Activities of Whole Plant Extracts of *Rhynchosia heynei* Wight & Arn: An Endemic Medicinal Herbaceous Under Shrub of Eastern Ghats of India

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This study reports the antioxidant and anticancer potential of *Rhynchosia heynei*, a widely used plant in traditional medicine, against various cancerous cell lines. The study found that *R. heynei* exhibited significant radical scavenging activity, indicating its potential as a natural antioxidant compound to guard against oxidative stress-induced diseases, including cancer. The plant extracts also displayed selective cytotoxicity towards various cell lines, with the most potent cytotoxicity exhibited by the ethanol extract towards the MCF-7 cell line and diethyl ether extract towards the HCT-116 cell line. The hexane extract exhibited substantial cytotoxic activity against HepG2, HCT-116 and L6 cell lines. However, the aqueous extract of *R. heynei* was the least cytotoxic of all the cell lines employed. Overall, the cytotoxic activity of *R. heynei* extracts was majorly selective towards MCF-7 and HCT-116. The findings suggest that *R. heynei* could be a potent natural product for developing anticancer drugs with fewer side effects. Further, *in vitro* and *in vivo* studies are required to determine the exact mechanism of action of the plant's potential anticancer activity.

Keywords: *Rhynchosia heynei*, Antioxidant, Anticancer, Selective cytotoxicity, SRB assay, Natural compounds, Phytochemicals.

INTRODUCTION

Cancer is a disease that affects people all over the world and does not recognize international borders. Pandemic caused delays in cancer detection and treatment due to the closures of healthcare facilities, shifts in work and anxiety over possible exposure to COVID-19 [1]. About 112 of 183 countries will have cancer as their primary or secondary cause of death in 2019, according to the World Health Organization (WHO). The International Agency for Research on Cancer (IARC) has just released estimates for the worldwide incidence of 36 different cancer types across 185 different nations [2]. In 2020, GLOBOCAN predicts that there will be around 19.3 million new cases of cancer diagnosed worldwide, with an estimated 10 million deaths attributable to the disease. Lung, prostate and colon cancer are more common in men, while breast, colon and lung cancer are more common in women.

Female breast cancer has surpassed lung cancer to become the most often diagnosed malignancy with a projected 2.3 million new diagnoses (11.7%), followed by lung (11.4%), colorectal (10.0%), prostate (7.3%) and stomach (5.6%) malignancies. Lung cancer was still the leading cause of cancer death, accounting for an estimated 1.8 million deaths (18%), followed by malignancies of the colon, liver, stomach and breast in women. Mortality rates also differed by sex, but were generally two to three times higher in developed countries than in those still in transition [3]. Increases in cancer rates can be attributed to a number of factors, including rapid urbanization, lifestyle variables and longer life expectancy. More than half of all new cancer cases and over 60% of all cancer deaths occur in Asia. Cancer incidences are 22.8% higher in Europe than in the Americas, whereas cancer mortality is 19.6% higher in Europe than in the Americas [2]. There are expected to be 1,958,310 new cases of cancer and 609,820 deaths from cancer in the United States in 2023. Furthermore, over the next two decades, the number of new cancer cases is projected to rise by 50% [4] and by 2040, up to 28 million people will get cancer each year and about 16 million will die from it [5,6].

Native to the forests of the Tirumala hills, the Seshachalam biosphere reserve, the Chittoor district and the Eastern Ghats of India, *Rhynchosia heynei* Wight & Arn. (Fabaceae) is known locally as Adavi Yulava [7]. The tribal people who live in the hills of the Eastern Ghats have traditionally relied on this plant to treat aches and pains associated with rheumatism, arthritis and skin conditions [8]. It is a wild relative of pigeon peas (Cajanus), beans (Phaseolus) and grains (Vigna). *R. heynei* is in the rare, endemic and vulnerable categories, according to the IUCN Red List of threatened species [9]. The leaves of *R. heynei* have been widely used as antimicrobial agents for rheumatic pain, arthritis and skin diseases by Adivasi tribal people (Chenchu and Lambada) inhabiting the hill ranges of the Eastern Ghats [10,11]. It was also scientifically reported in a study that the plant possessed potential antimicrobial properties, especially against *Staphylococcus aureus*, *Micrococcus luteus* and *Pseudomonas aeruginosa.* Phytochemical research on *R. heynei* have been conducted in a very limited number of studies. Naturally occurring flavones and flavone glycosides like apigenin, luteolin, vitexin, isovitexin, vicenin-2, orientin, isoorientin and lucenin-2 were isolated from *R. heynei* [12]. Several studies have reported the anticancer potential of these reported phytochemicals of *R. heynei.* Apigenin's potential to influence several cell signalling pathways, such as tumour suppressor genes, angiogenesis, apoptosis, cell cycle, inflammation, apoptosis, PI3K/AKT, NF-B, MAPK/ERK and STAT3 is verified [13]. Luteolin substantially suppresses tumour formation by inactivating multiple signals and transcription mechanisms required by cancer cells [14]. *In vitro* and *in vivo* investigations indicate that vitexin and isovitexin are the chemopreventive substances with action against different malignancies *via* pro-apoptotic and/or autophagic mechanisms [15]. Hence, the present study sought to investigate and confirm the anticancer property of *R. heynei* in various cell lines.

EXPERIMENTAL

The entire *Rhynchosia heynei* plant was collected, shade dried and ground to powder. 5 g of sample powder was obtained and mixed with 50 mL of ethanol, 50 mL of diethyl ether, 50 mL of hexane and 50 mL of demineralized water, separately by successive solvent extraction procedure. In case of ethanol, dimethyl ether and hexane, the sample mixture was incubated on a rocker shaker for 24 h. While in case of aqueous extraction, the sample combination was reduced to one-fourth of its initial volume by boiling. The extract was then filtered using Whatman filter paper 1 and fully dried in an oven at 40 ºC. The extract was collected in a microcentrifuge tube and kept at 4 ºC.

DPPH radical scavenging assay: In a 96-well plate, 10 µL of the test stock solution (0-2500 µg/mL) was mixed with 0.2 mL of 0.2 mM DPPH solution. The reaction was carried out in triplicates, with duplicates of the blank containing 0.2 mL DMSO/methanol and 5 µL test substance at various concentrations (0-2500 µg/mL). The plate was then incubated in the dark for 30 min. The decolorization was measured using a microplate reader (iMark, BioRad) at 495 nm at the end of incubation. Ascorbic acid was employed as a reference control [16]. The scavenging activity was expressed as a percentage inhibition with respect to control and the IC_{50} values were calculated [17].

Superoxide radical scavenging assay: The nitroblue tetrazolium (NBT) was converted by the superoxide radical into NBT diformazan in this experiment. When reduced with a superoxide anion, the extremely water-soluble tetrazolium salt used by SOD yields a water-soluble formazan colour. SOD inhibits xanthine oxidase activity, which is a factor in the rate of O_2-based reduction [18]. In this radical technique, nitro blue tetrazolium (NBT) is reduced to a purple formazan by the superoxide radicals produced by non-enzymatic phenazine methosulfate/nicotinamide adenine dinucleotide (PMS/NADH). The reaction mixture contained 20 mM phosphate buffer (pH 7.4), 73 mM NADH, 50 mM NBT and 15 mM PMS. It was added to 1 mL of the reaction mixture and incubated for 10 min at room temperature. The absorbance at 562 nm was measured against a blank using a UV-visible spectrophotometer. The experiment was conducted thrice and the data was expressed as percentage inhibition.

By plotting the proportion of scavenging activity against the logarithm of concentrations on a non-linear regression curve, the 50% inhibitory concentration (IC_{50}) values of extracts or isolated compounds were discovered [18]. A positive control was ascorbic acid. The findings of each test were carried out in triplicate and are shown as mean standard deviation (SD).

Cells culture: The cell lines MCF-7 (human breast cancer), HaCaT (immortalized human keratinocytes), HepG2 (liver cancer), A549 (lung carcinoma epithelial cells), HCT-116 (human colorectal carcinoma) and L6 (skeletal muscle cell lines) were acquired from the American Type Culture Collection (Rockville, USA) for conducting the study. All the cells (8000 cells/well) were cultured in 96-well plates for 24 h in Dulbecco's Modified Eagle's Medium (DMEM, Catalogue No. AT149-1L) supplemented with 10% foetal bovine serum (FBS) and 1% antibiotic solution at 37 ºC with 5% CO_2.

Sulforhodamine B (SRB) assay: The sulforhodamine B (Ottokemi, Catalogue No. 3520-42-1) assay was employed to assess the cytotoxicity of the extracts on cell lines. The cells (8000 cells/well) were grown for 24 h in DMEM media supplemented with 10% FBS and 1% antibiotic solution in a 96-well plate at 37 ºC with 5% CO_2. The following day, cells were treated with various doses ranging from 1-1000 µg/mL (various concentrations were made in an incomplete medium.) After a 24 h incubation period, each well received 100 µL of trichloroacetic acid (TCA, 10%, Fisher Scientific 28444) and was incubated for 1 h before being rinsed with demineralized water and air dried at room temperature. Each well was treated with SRB solution (final concentration of 0.04%) and left for 1 h. After 1 h of incubation, the plate was washed with 1% (v/v) acetic acid to remove unbound dye and air-dried at room temperature. Tris-base solution (pH = 10.5) was poured into the well and orbitally shaken for 10 min to solubilize the protein-attached dye before being read at 510 nm in an Elisa plate reader (iMark, Biorad, USA) [19].

Statistical analysis: All experiments were performed in triplicate and the results were expressed as mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

Among *Rhynchosia* genus plants, *R. heynei* is one of the most widely used in traditional medicine. Furthermore, it is widely apparent that study is being conducted to find naturally based anti-proliferative and chemo-preventive treatment options that can serve as substitutes for chemically synthesized medications, which are potentially less toxic and have fewer side effects.

Antioxidants operate as a defensive mechanism in the body, preventing chronic illnesses by lowering cellular oxidative damage produced by free radicals. In the case of cancer pathophysiology, upregulated free radicals and induced oxidative stress play a major role, involving several pathways. Hence, initially, the antioxidant property of *R. heynei* was determined through the *in vitro* radical scavenging activity exhibited by extracts in the DPPH assay and superoxide radical scavenging assay.

Radical scavenging activity: The radical scavenging activity of all the extracts was estimated using a DPPH assay. All the extracts displayed radical scavenging activity in a concentration-dependent manner. The IC_{50} value of hexane extraction of *R. heynei* was 27.37 µg/mL, which was close to ascorbic acid (IC_{50} value of 8.827 µg/mL), followed by diethyl ether (IC_{50} value of 159.3 µg/mL), aqueous (IC_{50} value of 445.4 µg/mL) and ethanol extract whose IC_{50} was found to be 573.20 µg/mL (Table-1).

The results indicated that *R. heynei* possessed significant antioxidant activity since all the extracts possessed concentration dependent radical scavenging properties. When compared to the standard ascorbic acid, the IC_{50} values of the extracts were in the following order: hexane, diethyl ether, aqueous and ethanol extracts (Fig. 1). However, the ethanolic extract of *R. heynei* exhibited the least radical scavenging property and the IC_{50} value was above the concentration range employed for performing the assay.

Superoxide radical scavenging assay: The superoxide radical-quenching assay was used to measure how well all of the extracts got rid of free radicals. Fig. 2 shows the radical IC_{50} values of the activity of scavenging in comparison. All the extracts showed a concentration-dependent ability to get rid of free radicals. The IC_{50} value of hexane extraction of *R. heynei* was 13.68 µg/mL, which was close to ascorbic acid's value of 8.827 µg/mL. This was followed by diethyl ether (IC_{50} value of 168.9 µg/mL), aqueous (IC_{50} value of 385.6 µg/mL) and ethanol extract, whose IC_{50} is 482.50 µg/mL.

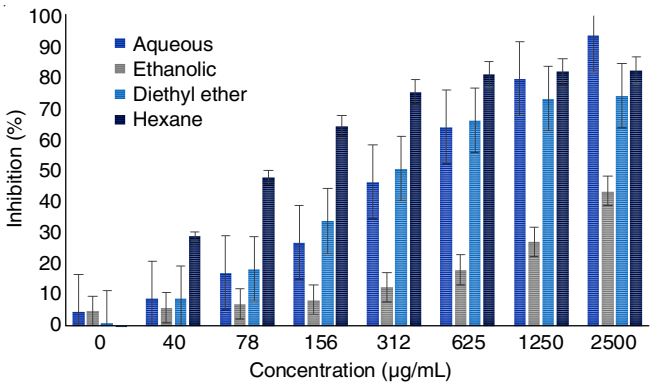

Fig. 1. Radical scavenging activity of different extracts of *R. heynei*. The data is expressed as mean ± SEM, (n = 3)

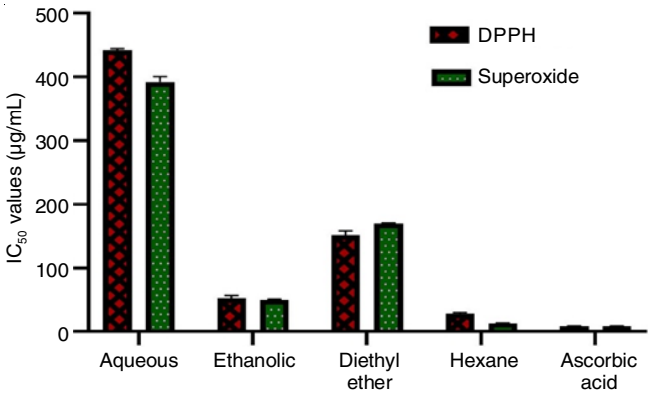

Fig. 2. Comparative bar graph showing IC_{50} values of different extracts

Cytotoxic potential of *R. heynei*: The cytotoxicity potential of *R. heynei* was estimated against cell lines like MCF-7, HaCaT, A549, HepG2, HCT-116 and L6 using the SRB assay. The results are presented in Table-2. The comparative IC_{50} values are depicted in Fig. 3. All four extracts exhibited dose-dependent inhibition of cell viability in all the cell lines, with the aqueous extract being the least active and the hexane extract

TABLE-1
PERCENTAGE OF INHIBITION AND IC_{50} VALUES OF EXTRACTS USING DIFFERENT ANTIOXIDANT METHODS

Sample	Percentage of inhibition (%) at different concentrations*							IC_{50} values (µg/mL)
	40 µg/mL	78 µg/mL	156 µg/mL	312 µg/mL	625 µg/mL	1250 µg/mL	2500 µg/mL	
DPPH free radical								
Aqueous	9.05 ± 0.73	17.24 ± 1.27	27.08 ± 0.62	46.64 ± 0.18	64.29 ± 0.40	79.81 ± 0.80	93.82 ± 1.84	445.90
Ethanolic	5.93 ± 1.44	7.15 ± 0.74	8.48 ± 1.63	12.49 ± 0.55	18.17 ± 0.55	27.16 ± 0.16	43.58 ± 0.67	573.20
Diethyl ether	8.91 ± 0.71	18.51 ± 0.56	37.87 ± 0.77	50.84 ± 1.77	66.44 ± 0.72	73.35 ± 0.91	74.35 ± 1.20	159.30
Hexane	29.04 ± 0.88	47.96 ± 1.71	64.69 ± 2.10	75.60 ± 0.31	81.32 ± 0.80	82.14 ± 0.14	82.61 ± 0.23	27.37
Superoxide free radical								
Aqueous	8.09 ± 1.25	16.02 ± 0.32	25.11 ± 1.21	42.21 ± 0.35	60.89 ± 0.69	72.31 ± 0.92	89.16 ± 0.64	385.60
Ethanolic	3.98 ± 0.95	5.21 ± 0.24	6.51 ± 0.98	10.49 ± 0.55	14.17 ± 0.55	24.16 ± 0.16	40.58 ± 0.67	482.50
Diethyl ether	9.32 ± 1.35	20.35 ± 0.21	39.91 ± 0.32	52.51 ± 0.32	69.624 ± 1.54	76.62 ± 0.62	79.29 ± 0.21	168.90
Hexane	26.65 ± 1.69	42.21 ± 1.21	58.35 ± 0.98	68.32 ± 0.59	75.92 ± 0.28	79.62 ± 0.62	80.29 ± 0.72	13.68
	1.32 µg/mL	2.65 µg/mL	5.31 µg/mL	10.60 µg/mL	21.30 µg/mL	42.50µg/mL	85 µg/mL	8.827
Ascorbic acid	5.75 ± 0.91	13.98 ± 0.79	28.94 ± 1.30	50.99 ± 1.24	65.62 ± 0.84	82.32 ± 0.46	85.90 ± 0.13	

*n = 3, mean ± SD

TABLE-2
PERCENTAGE OF INHIBITION AND IC_{50} VALUES OF EXTRACTS USING DIFFERENT ANTIOXIDANT METHODS

Conc.	Percentage inhibition wrt control ± SD							
	Ethanol	Aqueous	Hexane	Diethyl ether	Ethanol	Aqueous	Hexane	Diethyl ether
	MC7				HaCaT			
0	100.00 ± 4.33	100.00 ± 2.40	100.00 ± 8.34	100.00 ± 4.97	100.00 ± 3.53	100.00 ± 5.79	100.00 ± 3.16	100.00 ± 8.40
1	83.08 ± 19.44	86.85 ± 4.85	77.50 ± 7.50	88.66 ± 7.10	105.27 ± 1.53	113.70 ± 2.79	106.72 ± 9.53	110.70 ± 6.54
10	70.66 ± 12.28	85.39 ± 3.50	67.71 ± 7.81	74.02 ± 14.25	102.43 ± 5.63	119.75 ± 4.70	104.88 ± 6.56	102.42 ± 2.41
50	50.74 ± 5.65	83.08 ± 6.87	58.51 ± 7.41	60.41 ± 5.40	97.56 ± 4.88	118.14 ± 5.79	90.02 ± 5.34	88.28 ± 3.12
100	16.27 ± 8.66	74.11 ± 6.93	50.29 ± 2.42	43.71 ± 4.04	86.20 ± 3.71	114.51 ± 4.41	73.11 ± 3.72	67.67 ± 6.54
250	4.71 ± 3.50	56.78 ± 6.13	37.57 ± 5.82	25.77 ± 8.09	71.80 ± 0.66	109.47 ± 3.11	28.30 ± 3.72	53.33 ± 3.36
500	5.78 ± 1.90	52.81 ± 2.19	18.78 ± 5.82	10.10 ± 4.73	62.06 ± 4.02	104.63 ± 4.13	25.45 ± 5.25	42.82 ± 4.40
1000	12.63 ± 3.59	38.20 ± 8.80	5.28 ± 1.37	1.85 ± 2.27	41.38 ± 7.08	76.00 ± 1.52	23.01 ± 4.62	39.19 ± 4.87
	A549				Hep G2			
0	100.00 ± 4.61	100.00 ± 6.33	100.00 ± 3.78	100.00 ± 5.95	100.0 ± 14.26	100.00 ± 6.48	100.00 ± 8.09	100.0 ± 27.64
1	94.66 ± 6.25	98.43 ± 7.38	100.61 ± 6.65	95.16 ± 2.20	86.80 ± 3.14	98.64 ± 8.91	81.74 ± 13.92	90.51 ± 9.86
10	93.00 ± 7.16	94.68 ± 1.19	99.69 ± 7.83	92.90 ± 3.41	90.85 ± 2.23	77.25 ± 4.19	63.09 ± 6.75	73.08 ± 9.06
50	90.66 ± 16.25	96.25 ± 1.61	92.35 ± 6.84	64.19 ± 13.21	86.18 ± 2.37	70.62 ± 5.05	60.90 ± 3.56	53.27 ± 2.53
100	47.33 ± 2.43	93.43 ± 2.13	25.99 ± 13.03	27.74 ± 3.31	80.54 ± 6.26	83.58 ± 1.80	42.51 ± 11.58	31.65 ± 5.43
250	47.00 ± 5.03	87.18 ± 3.44	2.14 ± 2.08	27.09 ± 10.76	77.04 ± 8.28	75.16 ± 6.53	7.03 ± 1.98	26.77 ± 2.98
500	46.33 ± 6.83	76.25 ± 1.02	3.97 ± 1.53	24.83 ± 4.98	62.95 ± 11.69	70.86 ± 9.10	4.51 ± 0.98	25.94 ± 1.36
1000	31.66 ± 15.60	47.50 ± 4.62	6.42 ± 2.08	33.22 ± 2.20	49.27 ± 6.77	56.23 ± 8.29	2.90 ± 1.23	42.53 ± 10.97
	HCT 116				L 6			
0	100.00 ± 4.92	100.00 ± 4.68	100.00 ± 7.37	100.00 ± 2.84	100.00 ± 2.45	100.00 ± 3.72	100.00 ± 5.41	100 ± 7.16
1	90.01 ± 1.02	93.56 ± 5.44	98.34 ± 7.26	94.42 ± 8.02	97.49 ± 7.31	99.37 ± 3.78	99.28 ± 7.25	98.04 ± 6.66
10	70.23 ± 1.60	80.14 ± 5.38	90.04 ± 4.31	94.03 ± 3.78	96.42 ± 4.73	86.91 ± 8.65	88.88 ± 7.68	87.90 ± 2.23
50	64.06 ± 2.98	78.86 ± 2.64	89.00 ± 7.02	35.96 ± 2.54	86.74 ± 6.72	80.06 ± 8.89	57.34 ± 6.72	77.78 ± 7.47
100	55.71 ± 2.47	70.95 ± 7.57	-3.29 ± 2.42	10.19 ± 1.31	77.42 ± 3.51	75.39 ± 4.82	42.65 ± 12.24	58.17 ± 8.12
250	48.63 ± 9.15	70.77 ± 4.26	-3.57 ± 11.85	9.80 ± 0.73	59.86 ± 2.45	67.29 ± 2.39	31.89 ± 7.25	49.02 ± 8.44
500	9.61 ± 5.84	68.93 ± 5.64	-8.78 ± 16.32	8.46 ± 2.03	48.02 ± 1.17	59.50 ± 5.51	9.67 ± 0.71	42.48 ± 4.33
1000	9.61 ± 7.45	58.27 ± 9.94	-2.28 ± 1.65	2.73 ± 12.12	34.05 ± 1.37	53.89 ± 1.19	6.09 ± 2.15	37.25 ± 6.45

*n = 3, mean ± SD

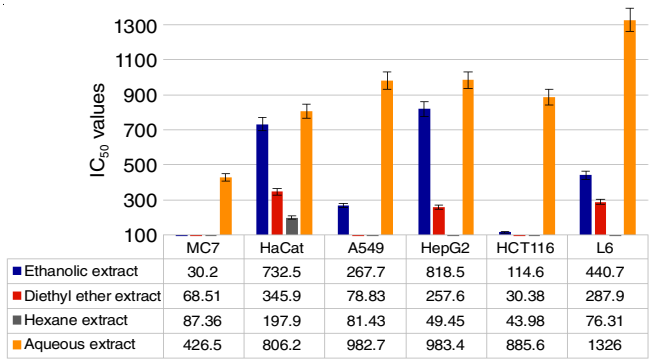

Fig. 3. Radical scavenging activity of different extracts of *R. heynei*. The data is expressed as mean ± SEM, (n = 3)

being the most active. Hexane extract displayed a significant cytotoxic activity towards HCT-116 (IC_{50} value of 43.98 µg/mL) and HepG2 cell lines (IC_{50} value of 49.45 µg/mL), followed by the A549 (IC_{50} value of 81.43 µg/mL) cell line. However, it was not effective against cell lines like HaCaT and MCF-7. Diethyl ether extract exhibited potent cytotoxic activity against the cell line HCT-116 (IC_{50} value of 30.38 µg/mL), followed by MCF-7 (IC_{50} value of 68.51 µg/mL) and A549 (IC_{50} value of 78.83 µg/mL). Further, an ethanolic extract of *R. heynei* displayed a significant cytotoxicity towards the MCF-7 cell line with an IC_{50} value of 30.2 µg/mL, *albeit it* was not effective against other cell lines. All the extracts could not display cytotoxicity towards the HaCaT cell line.

All the extracts of *R. heynei* displayed concentration-dependent cell viability inhibition in all five cell lines empanelled for assessment. However, the potency of cytotoxicity exhibited by the extracts was selective for a particular cell line. The most potent cytotoxicity was exhibited by an ethanol extract of *R. heynei* with an IC_{50} of 30.20 µg/mL towards the MCF-7 cell line and was not effective against other cell lines. Diethyl ether extract of *R. heynei* exhibited potent cytotoxic activity selectively towards the HCT-116 cell line (IC_{50} of 30.38 µg/mL), followed by moderate cytotoxicity towards the MCF-7 and A549 cell lines. While the hexane extract of *R. heynei* exhibited substantial cytotoxic activity against HCT-116 and HepG2 cell lines with IC_{50} values of 43.98 µg/mL and 49.45 µg/mL, respectively, On the other hand, in L6, A549 and MCF-7 cell lines, the cytotoxic activity of hexane extract was moderate, with IC_{50} values of 76.31 µg/mL, 81.43 µg/mL and 87.36 µg/mL, respectively. Hexane extract was the only fraction of *R. heynei* that exhibited selective activity towards the HepG2 cell line. Further, among all the four extracts, the aqueous extract of *R. heynei* was least cytotoxic towards all the cell lines employed. Additionally, none of the extracts were able to exhibit potent inhibition against the HaCaT cell line. Overall, the cytotoxic activity of *R. heynei* was majorly selective towards MCF-7 and HCT-116.

Conclusion

The current study found that the plant *Rhynchosia heynei* had antioxidant and anticancer activity. The plant exhibited

significant radical scavenging activity, which is an important property of a natural compound that plays an important role in a variety of diseases, including cancer. Further, the plant also displayed selective cytotoxicity towards various cell lines and it was dependent on the fraction of extract. Additional *in vitro* and *in vivo* studies are required to determine the exact mechanism of action of the plant's potential anticancer activity.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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