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IN VITRO ANTIOXIDANT AND ANTICANCER ACTIVITY OF THE FRUIT EXTRACT OF *RIVINA HUMILIS* L. (RED PIGEON BERRY)

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ABSTRACT

The purpose of this study was to evaluate the cytotoxic and antioxidant properties of a hydromethanolic extract of *Rivina humilis* L fruits extract (HMERH) on human cancer cell lines and various *in vitro* models. HMERH concentrations ranging from 5 to 400mg/ml were utilized in several assay methods such as hydroxyl radicals, DPPH, superoxide radical scavenging activity, metal chelating, and nitric oxide radical test, ABTS, and reducing power assay. All antioxidant assays employed ascorbic acid as the standard. Its short-term cytotoxicity on EAC and DLA cells was determined using the trypan blue dye exclusion method, as well as the lactate dehydrogenase (LDH) leakage assay and in vitro cytotoxicity on MEF-L929, DU-145, and PC-3 cells using the MTT assay. The total antioxidant potential was also determined using the phosphomolybdenum test. HMERH demonstrated considerable cytotoxicity against all cancer cell lines examined. The findings of the free radical scavenging activities demonstrated a concentration-dependent and antiradical activity caused by the reduction of ABTS, DPPH, NO, OH, and SO radicals to non-radical form. The findings reveal that antioxidants and cytotoxic agents have potent antioxidant and cytotoxic effects on cancer cell lines. Hence, this fruit extract has the potential to be a natural antioxidant and anticancer drug source, which could be valuable in the development of new anticancer therapies.

Keywords: *In-vitro*, anticancer, antioxidant, *Rivina humilis* L, Lactate dehydrogenase, MTT assay

INTRODUCTION

In the world, Cancer is a serious disease that has high morbidity and mortality rate than other diseases. Among cancer, breast cancer is the most important cause of high mortality rate in females (**Ma *et al.*, 2006**). Even though surgery, chemotherapy, and radiotherapy are the mode of treatments available to save the lives of patients but some extent they may cure cancer partially however severe side effects from drug treatment and the recurrence rate of cancer are again factors of concern to control or completely cure of cancer and therefore the demand for developing cancer therapies from different sources other than the synthetic chemotherapeutic drugs essential to overcome the current issues (**Raju *et al.*, 2021**). Plant-derived natural compounds such as vinblastine, vincristine, and camptothecin are low in toxicity, have great target specificity, and are widely used in cancer treatment (**Liang *et al.*, 2017**). One such medicinally important candidate is the bright red pigeon berry *Rivina humilis* L., family Phytolaccaceae. It's an herbaceous bushy wild plant found in shaded soils. It contains an important phytoconstituent called Betalains. It is a natural antioxidant, active against oxidative stress-related diseases such as inflammation, atherosclerosis, ischemia, asthma, diabetes, cardiovascular diseases, and viral infections. This plant was traditionally used for various diseases such as gonorrhea and jaundice (**Khan *et al.*, 2011**). Researchers identified 14 phytoconstituents by GC-MS from the ethanol extract of the stem of *R. humilis* with different biological activities (**Mujeera *et al.*, 2012**). The methanol leaf extract of *R. humilis* was shown to have strong free radical scavenging activity *(in-vitro*) and a greater amount of flavonoids and tannins. These phytoconstituents and metabolites exhibit a wide variety of biological functions, including cancer prevention (**Kavitha *et al.*, 2019**). A comprehensive review of the literature provided no difference between the antioxidant and anticancer effects of *R. humilis* fruit extracts. Hence, an attempt was made with a hydroethanolic extract of *R. humilis* fruits on various *in-vitro* models of antioxidant and anticancer activity.

MATERIALS AND METHODS

Plant materials

Fruits of *R. humilis* L (see Figure) were collected in October 2020 from Marthandam, Tamil Nadu, and Kayamkulam, Kerala, India. Professor Dr. Sandhya P, HOD, Department of Botany, NSS College, Pandalam, identified and authenticated the collected whole plant material. Herbarium BOHDOS-2/27/11/2020 was deposited at the Department of Botany, NSS College, Pandalam, Kerala, India.

Figure1 R. *humilis* L (fruits)

Preparation of the extract

The dried powdered form of *R. humilis* fruit material was successively extracted by cold maceration using water and methanol (70:30) for 72 hours with occasional shaking until the soluble matter had dissolved or completion of extraction. After 72 hours, the mixture was strained through a muslin cloth and squeezed to remove all the remaining liquid. A rota evaporator was used for the solvent recovery (**Asirvatham *et al.*, 2022**). The crude extract was named "hydromethanolic extract of *R. humilis*" (HMERH) and was used to study the antioxidant as well as anticancer potential in different *in-vitro* models.

Procurement of cancer cell-lines

The National Centre for Cell Science (NCCS) in Pune, India, provided the MEF-L929, DU-145, and PC-3 cells. Amala Cancer Research Centre, Thrissur, Kerala, India, supplied Dalton's Ascitic Lymphoma (DAL) and Ehrlich Ascitic Carcinoma (EAC) for this study.

Antioxidant assays (*In-vitro* models)

All the assays had been accomplished in triplicate and observed the methods of **Asirvatham *et al.*, 2013**.

DPPH (1, 1 diphenyl 2, picrylhydrazyl) Assay

1 ml of HMERH (5 to 400mg/ml) at various concentrations was mixed to 1 ml of DPPH in methanol 1mM). Instead of plant extract, an equivalent volume of methanol was added in the control test tube. After 20 minutes, under 517nm the absorbance was measured. The reference standard was ascorbic acid.
Inhibition of DPPH (%) = (A 0 – A 1 / A 0) × 100, where A 0 is the absorbance of the control and A1 is the absorbance of the test.

Hydroxyl radical scavenging assay

The Fenton reaction occurred when a reaction mixture of Fe^{3+}, ascorbate, EDTA, and H_2O_2 was added to form hydroxyl radicals (*in-vitro*). The formed hydroxyl radicals interact with dimethyl sulphoxide to form a product is called formaldehyde, which exhibited a dark yellow color with Nash reagent, and whose color intensity was measured spectrophotometrically at 412nm against blank. The reference standard used was ascorbic acid.
Hydroxyl radical scavenging assay (%) = 1-(Differences in absorbance of test sample / Difference in absorbance of blank) X 100

Nitric oxide (NO) radical scavenging

In phosphate buffer pH 7.4, 5 mM sodium nitroprusside was prepared, and 0.3 ml was added with 1 ml of various concentrations of HMERH (5 to 400mg/ml). The test tubes with the above content were incubated at 25^0C for a period of 5 hours before adding 0.5 ml of Griess reagent. The resultant product absorbance was measured at 546 nm. Ascorbic acid was used as a reference standard.
Inhibition (%) = (A 0 – A 1 / A 0) × 100, where A 0 is the absorbance of the control and A1 is the absorbance of the test.

Metal chelating assay

With 1 ml of 2mM $FeCl_2$, various concentrations of HMERH (5 to 400mg/ml) were added. BY adding 1 ml of ferrozine (5 mM), the reaction was initiated and after 10 minutes, color intensity was measured spectrophotometrically at 562 nm against blank. Ascorbic acid was used as a reference standard.
Inhibition (%) = (A 0 – A 1 / A 0) × 100, where A 0 is the absorbance of the control and A1 is the absorbance of the test.

Reducing power assay

1 ml of HMERH (5–400mg/ml) was added with 2.5 ml of phosphate buffer and 2.5 ml of 30 mM potassium ferric cyanide and the test tubes were incubated at 50°C for duration of 20 minutes. The reaction mixture was then treated with 2.5 ml of CH_3COOCl_3 (600 mM) and then resultant mixture was centrifuged for 10 minutes at 3000 rpm. 2.5 ml of supernatant was combined with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (6 mM), and the color intensity was measured at 700 nm. The reference standard used was ascorbic acid.
Inhibition (%) = (A 0 – A 1 / A 0) × 100, where A 0 is the absorbance of the blank and A1 is the absorbance of the test.

Superoxide scavenging assay

Addition of 0.5 ml of NBT to 0.5 ml of NADH generates superoxide anion radicals, in the same test tube add 1.0 ml of various concentrations of HMERH (5 to 400mg/ml), and 0.5 ml Tris - HCl buffer (16 mM, pH 8.0). The reaction was initiated by adding 0.5 ml Phenazine methosulphate (0.12 mM) to the mixture, which was then incubated at 25°C for duration of 5 minutes. The absorbance of the test and the blank were measured at 560 nm. The reference standard used was ascorbic acid.
Inhibition (%) = (A 0 – A 1 / A 0) × 100, where A,0 is the absorbance of the blank and A1 is the absorbance of the test.

ABTS cation radical scavenging assay

In a test tube containing ABTS radical (0.3 ml), 1.7 ml of phosphate buffer, and 0.5 ml of different concentrations of HMERH (5 to 400mg/ml), allowed to reacts for few minutes. Blank was performed without drug. The absorbance was measured at 734 nm. Ascorbic acid was employed as a control.
Inhibition (%) = (A0 – A1 / A0) × 100, where A0 is the absorbance of the control and A1 is the absorbance of the test.

Phosphomolybdenum (PM) assay

Ghagane et al. (2017) described the PM assay for determining total antioxidant activity. 1 ml of a different concentration of HMERH (5 to 400mg/ml) fruit extract (0.1%) was added to a set of three test tubes with 1 ml of molybdate reagent and 3 ml of distilled water and were incubated at 95 °C for 90 minutes. The absorbance was measured at 695 nm after incubation. The reference standard used was ascorbic acid (**Ghagane *et al*., 2017**).

In- vitro anticancer activity

Trypan blue exclusion method

Different concentrations of HMERH (5 to 400mg/ml) in which 100 µl of fruit extract was added with 800 µl of phosphate buffer saline and 100µl ($1X10^6$ in 1 ml) of DAL (**Asirvatham *et al*., 2013**). The same approach was followed with the EAC cell line. Each extract concentration was tested for three times. All the test samples were incubated at 37°C for 30 minutes. About 100 µl of trypan blue dye was added to all the test tubes followed by the dead cells was counted under 10X in a light microscope (Nikon, Tokyo Japan). The following formula was used to calculate the percentage of cytotoxicity.
% of dead cells = Number of dead cells / Sum of dead cells and living cells × 100.

LDH (Lactate Dehydrogenase) leakage assay

LDH cytotoxicity detection kit wsa purchased from Sigma Aldrich Inc., USA, to determine the IC_{50}, different concentrations of HMERH (5 to 400mg/ml) fruit extracts were incubated with $1X10^6$ cell/ml in 100 µl of DAL and EAC cells separately in a 96 well plates and were incubated at 37°C for 4 hours (**Asirvatham *et al*., 2013**). To calculate cytotoxicity, blank and test samples were tested in triplicate and the mean ± SEM was recorded.

LDH leakage (%): [A] test / [A] control × 100.

Where [A] test is the absorbance of the test sample, and [A] control is the absorbance of the control sample.

MTT assay

Ghagane *et al*., 2017 described a MTT assay procedure for the subculture of MEF-L929, DU-145, and PC-3 cells. The MTT assay method was used to ensure cell viability. The cell count was adjusted so that each well contained $2X10^3$ cells/well in 100 µl MEF-L929, DU-145, and PC-3 cell suspensions and $2X10^6$ cells/ml in 100 µl DAL and EAC cell suspensions before performing an MTT assay. In the well-containing cell culture, different concentrations of HMERH (5 to 400mg/ml) fruit extracts were added and incubated for 72 hours. Standard drugs like paclitaxel (5-15 µg/ml) for MEF-L929, DU-145, PC-3, and 5- flurouracil (10-30 µg/ml) for DAL and EAC were added to the treatment well plate. Only one cell line possesses a well plate, which was also used as a control in the study. After 72 hours of incubation, 50 µl of MTT at 37 °C was added, which resulted in the formation of Formazan. It was dissolved in dimethyl sulphoxide (100 µL) and kept on a micro vibrator for 5 minutes before the absorbance at 540 nm was measured using an ELIZA reader. The percentage of cell growth inhibition was calculated as % cytotoxicity as per the following formula.
Cytotoxicity (%) = mean OD sample/ mean OD blank X 100
A double beam UV-VISIBLE spectrophotometer (UV-1800, Shimadzu, Japan) was used for measurement of absorbance.

RESULTS

Using various *in vitro* models, various concentrations of HMERH (5 - 400mg/ml) were tested to assess the property of the free radical scavenging effect, and the findings are shown in Table 1. Concentration-dependent percentage of inhibition was observed in this assay. Metal chelating and superoxide scavenging assays consumed lower concentrations of extract than ascorbic acid.

Table 1 *In vitro* antioxidant effect of HMERH fruits

S no.	In vitro Model	Extract Concentration Used (mg/ml)	IC_{50} Concentration of HMERH (µg)	IC_{50} Concentration of AA (µg)
1	DPPH	5, 10,20, 40, 80,100, 200 and 400	55 ±1.3	47 ±2.8
2	Hydroxyl radical scavenging activity		68 ±2.7	59 ±6.1
3	Nitric oxide radical scavenging		102 ±3.5	85 ±3.1
4	Metal chelating activity		88 ±4.2	98 ±5.5
5	Reducing power Assay		93 ±2.1	70 ±3.2
6	Superoxide scavenging		71 ±1.9	92 ±2.5
7	ABTS radical scavenging assay		127 ±7.2	82 ±1.6

Table 2 showed the in vitro anticancer activity of HMERH by the trypan blue dye exclusion method. The maximum cytotoxicity was found with 400mg/ml concentration (99 ± 1.2%) in DAL and 94±1.7% in EAC. It has shown a concentration-dependent cytotoxic effect. Similarly, % of cytotoxicity was calculated by LDH leakage assay which also showed concentration-dependent cytotoxicity, where 400mg/ml HMERH showed 92±1.4 percent cytotoxicity in the DAL cell line, whereas 88±2.1 percentage cytotoxicity was found with the EAC cell line.

Table 2 Effect of HMERH on % cytotoxicity by Trypan blue exclusion method and LDH leakage assay

Cell line	Name of extract	Concentration used (mg/ml)	% of cytotoxicity			
			Trypan blue exclusion		LDH leakage assay	
			%	IC_{50}	%	IC_{50}
DAL	HMERH	5	18±1.3	39	7±2.1	63
		10	23±1.5		16±1.6	
		20	38±2.4		38±1.7	
		40	51±1.6		45±1.2	
		80	72±1.5		64±2.4	
		100	87±2.1		73±1.5	
		200	91±2.5		89±2.1	
		400	99±1.2		92±1.4	
EAC	HMERH	5	13±1.2	34	8±1.3	80
		10	25±1.7		14±1.5	
		20	43±1.5		26±2.2	
		40	58±1.6		38±1.6	
		80	79±1.8		41±1.1	
		100	86±2.4		62±1.2	
		200	91±1.5		79±2.3	
		400	94±1.7		88±2.1	

The percentage of cytotoxicity produced by HMERH fruit extract on various cell lines is illustrated in Table 3. The IC_{50} value for the MEF-L929 cell line was 88±2.6, indicating 92 percent cytotoxicity. The IC_{50} value for the DU-145 cell line was 102±4.4, indicating 87 percent cytotoxicity. The IC_{50} value for the PC-3 cell line was 64±2.8, indicating 94 percent cytotoxicity. For the DAL cell line, the IC_{50} value was 58±1.58 with 97% cytotoxicity, whereas the EAC cell line showed 98% cytotoxicity and required an IC_{50} concentration of 65±2.7. The percentage of cytotoxicity with 50% inhibitory concentration was calculated where 58±1.5 µg/ml concentration was needed to produce half of cell death in DAL, which was the minimum concentration of extracts consumed out of five cell lines and showed 97% cytotoxicity. For the DU-145 cell line, 102±2.4 µg/ml concentration was needed to produce half of the cell death and produced 87% cytotoxicity, which was the least percentage when compared with other percentages of cytotoxicity, but almost all the extracts exhibit similar percentages of cytotoxicity as that of standard Paclitaxel and 5-Flurouracil.

Table 3 Cytotoxicity (%) of HMERH on various cell lines by MTT assay

Drug treatment	Concentration used (mg/ml)	MEF-L929		DU-145		PC-3		DAL		EAC	
		%	IC_{50}	%	IC_{50}	%	IC_{50}	%	IC_{50}	%	IC_{50}
HMERH	5, 10,20, 40, 80,100, 200 and 400	92±1.3	88±2.6	87±4.1	102±2.4	94±3.5	64±2.8	97±2.5	58±1.5	98±1.8	65±2.7
Paclitaxel	5, 10, 15	97±2.1	8±1.6	98±1.1	12±2.3	96±1.5	10±1.9				
5-Flurouracil	10, 20, 30							94±1.4	14±1.5	98±1.6	15±2.1

The total antioxidant potential of HMERH fruit extract was determined using a phosphomolybdenum assay, and the results are shown in Table 4 as concentration versus absorbance. A concentration-dependent increase in absorbance was found with HMERH fruit extract, where higher concentrations produced equivalent absorbance to that of standard ascorbic acid.

Table 4 Total antioxidant activity by phosphomolybdenum assay

Name of extract	Concentration used (mg/ml)	Absorbance (nm)
HMERH	5	0.325±2.65
	10	0.350±3.50
	20	0.357±2.95
	40	0.455±2.65
	80	0.550±2.58
	100	0.750 ±1.87
	200	1.09±0.51
	400	1.40 ± 0.52
Ascorbic acid	500	1.32±0.47

DISCUSSION

Antioxidant systems play a critical role in protecting cellular membranes and organelles from the harmful effects of active oxygen species. Certain non-enzymatic assays were used in this work to reduce free radical production, and the IC_{50} values are evaluated. The IC_{50} values were derived by interpolating the concentration vs. percentage inhibition of the extracts. It is defined as the concentration of extracts needed to reduce the absorbance to 50% (**Kurutas, 2016**). The DPPH assay is based on the measurement of free radical scavenging ability by an antioxidants. It is nitrogen centered free radical that can accept an electron form a stable molecule. When DPPH radicals react with reducing agent, electrons are paired off, resulting in the formation of the corresponding hydrazine. As a result, the solution loses color; the strength of color depends on the number of electrons consumed. HMERH, like Ascorbic acid, demonstrated DPPH radical scavenging activity. Similarly, the formation of superoxide radicals leads to a cascade formation of other reactive oxygen species in the cell. Superoxide anions produced from dissolved oxygen by the PMS/NADH coupling reaction reduce NBT in PMS/NADH-NBT systems. NBT reduction is the basic and important step in this method. The method is based on the production of superoxide radicals, which convert the reduced NBT to a blue formazon, intensity of the formed chromophore was measured at 560nm. The decrease in absorbance with antioxidants implies that superoxide anions are being consumed in the reaction mixture. In this work, HMERH demonstrated concentration-dependent free radical scavenging effects **(Balaji et al., 2015)**.

The NO radicals formed from the reaction between sodium nitroprusside and Griess reagent. The absorbance of the chromophore is measured at 546nm in the presence of scavengers. This scavenging power may be attributed to antioxidative principles (betalain) in the extracts, which compete with oxygen to react with nitric oxide, thereby inhibiting nitrite formation. HMERH exhibited concentration-dependent NO scavenging radical scavenging activity in our studies. In the reducing power assay, a colored complex was formed when potassium ferricyanide react with ferric chloridein presence of trichloroacetic acid, and the intensity of the color is measured at 700nm, where the presence of antioxidants in plant extracts prevent the reduction of the Fe^{3+}/ Ferricyanide complex to the ferrous form. In the current investigation, HMERH demonstrated concentration-dependent reducing power, similar to standard ascorbic acid. In a metal chelating assay, Ferrozine can quantitatively form complexes with Fe^{2+}. The absorbance of the Ferrozine-Fe^{2+} complex decreased linearly with different concentration of extracts. They act like chelating agents that form bonds with metals and reduce the redox potential and stabilize the oxidized form of the metal ion. In the current study, HMERH demonstrated concentration-dependent stability, similar to that of ascorbic acid (**Balaji *et al*., 2015**).

The most reactive radical in the ROS system is the OHl radical. It causes the lipid peroxidation of cell membrane, which results in the formation of malondialdehyde (MDA), which is both mutagenic and carcinogenic. In the current work, HMERH reduced hydroxyl radical formation in a concentration-dependent way. The ABTS assay technique is based on the interaction of ABTS with potassium persulphate to produce the ABTS radical ($ABTS^{+}$), a blue - green colored complex. The colored free radical is transformed back to colorless ABTS in the presence of extracts, and its absorbance at 734nm is measured. This method's free radical scavenging activity was concentration dependent (**Asirvatham *et al*., 2013**).

The trypan blue dye exclusion method was used to test anticancer activity *in-vitro*. Tryphan blue dye is used to identify thedead as well as the living cells. The assay is based on the principle of interaction of a negatively charged chromophore with a damaged cell membrane. Living cells with perfect cell membranes do not stain, but dead and injured cells do and can be seen under a microscope. HMERH exhibited a dose-dependent cytotoxic impact. In the present research, the percentage of LDH released increased as extract concentration increased. Lactate dehydrogenase is a glycoprotein isoenzyme which is released into the medium when cells die and their membrane integrity is lost. The release of LDH in the medium and the change (decrease) in activity over time suggest dead cells and loss of membrane integrity, and a value of 100% LDH release implies necrotic cell death. This is because the amount of LDH released into the extracellular environment equals the total LDH, showing that all cells died, and it may be assumed that the cells died via necrosis. The Lactate dehydrogenase release assay has the advantage of being simple to use (**Asirvatham *et al*., 2013**).

Surprisingly, natural products are the most important anti-cancer drugs. 75% of the antitumor compounds used in medicine to fight human cancer are natural resources. MEFL929, DU145, PC3, DAL, and EAC cell lines were selected to study the anticancer potential of HMERH on cancer cells. Both availability and sensitivity are the two main reasons for the selection of these cell lines. Preliminary screening of HMERH anti-cancer activity was performed on MEFL929, DU145, PC3, DAL, and EAC. Due to the occurrence of reactive oxygen species with a high level of change in metabolic rate, these cancer cells are functionally different from normal cells. Moreover, high levels of reactive oxygen species are required for cancer cell growth, proliferation, and cancer metastasis. Altogether, exceeding the ROS levels required by cancer cells can cause oxidative stress and, in some cases, death. Cancer cells, unlike normal cells, have been shown to have the limited antioxidant mechanisms needed to remove the surplus reactive oxygen species produced and also to prevent the cell damage that was associated with the free radical reaction. The antitumor (*invitro*) potential of the extracts exhibited here by the phytoconstituents present in the extract that alter the oxidation-reduction balance need for the existence of the above cell lines. This activity can induce or inhibit reactive oxygen species levels in cancer cell lines. The negligible toxicity of HMERH to normal cells may be due to the presence of strong free radical scavenging as well as anti-inflammatory mechanisms (**Ghagane *et al*., 2017**). *R. humilis* leaf extract has been reported to have antioxidants (**Kavitha *et al*., 2019**). DNA damage may be the underlying mechanism of extract-induced cell-specific toxicity. Generally, uncontrolled cell proliferation causes cell transformation through changes in multiple cell pathways, but different kinds of mutations exist in cancer studies and are unique in their pathway. Additional experiments are needed to find insight into the cellular processes targeted by the extract.

To determine the total antioxidant potential of HMERH fruit extracts, the phosphomolybdenum method was used, and a comparison with ascorbic acid was made. The HMERH fruit extracts had the highest antioxidant efficacy. The antioxidant activity of the HMERH fruit extracts was similar to that of ascorbic acid, confirming their high antioxidant capacity. *R. humilis* has a greater antioxidant effect due to the presence of active photo components such as tannins, polyphenols, etc (**Nelson *et al*., 2020**). The GC-MS method was used to identify 14 bioactive components in an ethanol extract of *R. humilis*. The presence of various phytoactive compounds in this plant is responsible for the pharmaceutical properties (**Mujeera *et al*., 2012**).

CONCLUSION

The study had established fruits of *R. humilis* retain acceptable antioxidant sources that fight against the free radicals generated from the different *in-vitro* chemical reaction models. Moreover, it possesses cytotoxic potential on the different human cancer cell lines. The antioxidant property of the phytoconstituents may play a major role in cytotoxic potential and should be investigated from the ethnopharmacological perspective as the best anticancer drug. This experimental result outcome gave appreciated information for the future progress of pioneering anti-cancer drugs from the medicinal plants.

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