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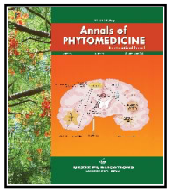

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Houttuynia cordata Thumb. extract for the treatment of triple negative breast cancer

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Abstract

A common herb in East Asia, *Houttuynia cordata* Thumb. (HCT) is known for its numerous biological properties, including its anticancer properties. However, its application in the treatment of breast cancer is largely unexplored. This is a heterogeneous group of malignancies originating from the epithelial cells lining the milk ducts and is the leading cause of cancer deaths in women worldwide, accounting for approximately 18% of all cancers in the female population. One of the aggressive and most challenging form of the breast cancer to treat is triple negative breast cancer (TNBC). The potential anticancer activities and mechanisms of HCT; however, not yet been investigated in TNBC cells. Therefore, the biological effects of HCT on the human breast cancer cell lines MDAMB231 (TNBC specific) was investigated in this work. In brief, we collected, authenticated and prepare aqueous extract of HCT and evaluated for its anticancer effects against TNBC. The extract was primarily analyzed for the presence/absence of various plant actives such as alkaloids, glycosides, *etc*. using reported protocols. Afterwards, the extract was analyzed using UV and FTIR for the confirmation. Further, the MTT assay was carried out to evaluate the dose dependent cell viability in MDAMB231 cells, which are specific to TNBC. In addition, we determined the hemocompatibility of the different concentration of the aqueous HCT extract using the standard protocols. Overall, these findings provide the basis for further investigation of HCT extracts against TNBC *in vivo*.

1. Introduction

Breast cancer with triple-negative status (TNBC), which affects two out of every 12000 women, is the most challenging form of the disease (Bianchini *et al.*, 2022; Zagami and Carey, 2022). Various factors contribute to the difficulty in treating TNBC, such as the absence of early diagnostic tools, the presence of negative hormonal expressions, the rapid progression of the disease, and the lack of a specific treatment (Fan and He, 2022; Luo *et al.*, 2022; Mehraj *et al.*, 2022). For the treatment of TNBC, oncologists rely on chemotherapy, radiotherapy, and hormonal therapy; however, the success of the treatment depends upon early detection and the survival rate decreases with advanced stage disease (Bou Zerdan *et al.*, 2022; Tarantino *et al.*, 2022). As well, the truckloads of adverse effects associated with chemotherapy and radiotherapy cannot be completely ignored (Arif *et al.*, 2022; Tan *et al.*, 2022; Uddin and Veeresh, 2020). Therefore, a more specific, chemotherapy and radiotherapy free treatment approach is warranted. In this context, plant-based therapy can be used as an alternative (Chaves, 2020).

Knowledge and use of plants as herbal medicines has occurred in various populations throughout human evolution, beginning when man was learning to select plants for food, and to relieve ailments and diseases (Arif *et al*., 2022; Khan and Ahmad, 2021; Shaikh *et al.*, 2022; Uddin and Veeresh, 2020). Natural products with a variety of pharmacological targets in numerous diseases have emerged as crucial sources for the development of novel drugs. *H. cordata* Thumb. (HCT), a medicinal plant native to North-East China and India, was recently explored in multiple of investigations (Kumari *et al.*, 2016). HCT possesses numerous pharmacological actions and includes thorough information on its morphology, distribution, phyto-chemistry, ethnopharmacological applications, and ethno-pharmacological uses. Besides, its antioxidant potential, it was also shown that HCT can be used to treat variety of cancer. As a result, the goal of this study was to use HCT aqueous extract to determine its potential application against the TNBC.

2. Materials and Methods

2.1 Plant material

Arial parts of HCT were collected locally from Guwahati, Assam State of India during the flowering session and utilized to get its aqueous extract.

2.2 Cell culture materials

MDA-MB-231 cell lines were received from National Centre for Cell Sciences (NCCS), Pune, India. The cell lines were suitably cultured following due protocols. Briefly, cells were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS), 1% L-glutamine, and 100 U/ml penicillin/streptomycin in aseptic conditions to maintain the sterility and at stored in 37°C in a humidified atmosphere containing 5% CO_2.

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2.3 Plant collection and authentication

HCT ariel parts were collected locally from Guwahati, Assam State of India during the flowering session and utilized to get HCT aqueous extract. The plant was authenticated (letter no ABRL/1074; Dr. Rajeev Sarmah, Dean, Faculty of Science, Down Town University, Assam) at Bioresources Center, Baihata Chariali, under Assam Science and Technology and Environmental Council, Govt. of Assam, India; after due submission of a specimen as herbarium.

2.4 Drying of plant material

After authentication, the obtained aerial parts were completely dried using hot air oven at 45°C-55°C for 36 h. The dried material was then subjected to blending followed by passing through sieve (sieve with mesh no 40) to finally obtained fine powder.

2.5 Preparation of HCT extract and % yield determination

For extraction, purified water and dried powder were macerated at a ratio of 10:1 for three times for 24 h, followed by filtration on Whatman paper grade 1 and drying in a hot air oven (Wang *et al.*, 2022). The finally obtained dried aqueous extract was stored at room temperature. After the preparation of extract, % yield of extract was determined using following formula:

$$\% \text{ Yield of extract } = \frac{\text{weigth of dried extract (g)}}{\text{wight of dried plant (g)}} \times 100$$

2.6 Preliminary phytochemical analysis of the HCT extract

Preliminary phytochemical screening is an important step to determine the secondary plant metabolites of any plant extracts. The extract obtained was then tested to determine the presence/ absence of various phytoconstituents as presented in Table 1. Different reagents were prepared for different test as presented in Table 1. The protocols used in the determination of the different plant principles are presented in Table 2.

Table 1: Reagent preparation for phytochemical screening

Reagents/solutions	Composition
1. Dragendroff's reagent	Stock solution: 5.2 g bismuth carbonate + 4 g sodium iodide + 50 ml glacial acetic acid, boiled for few min. After 12 h precipitated, sodium acetate crystals are filtered by sintered glass funnel; 40 ml filtrate + 160 ml ethyl acetate + 1ml distilled water (stored in amber-coloured glass bottle).Working solution: 10 ml stock solution + 20 ml acetic acid + distilled water to make final volume 100 ml
2. Hager's reagent	Solution of picric acid in water
3. Mayer's reagent	Solution A: 1.358 g mercuric chloride + 60 ml distilled water. Solution B: 5 g potassium iodide + 10 ml distilled water. Working solution: solution A + solution B + distilled water to make final volume 100 ml
4. Wagner's reagent	1.27 g iodine + 2 g potassium iodide + distilled water to make final volume 100 ml
5. Barfoed's reagent	30.5 g copper acetate + 1.8 ml glacial acetic acid
6. Seliwanoff's reagent	0.05 g resorcinol + 100 ml dilute HCl
7. Benedict's reagent	Solution A: 173 g sodium citrate + 100 g sodium carbonate + 800 ml water, dissolve and boil to make solution clear. Solution B: 17.3 g of copper sulphate dissolved in 100 ml distilled water. Working solution: Mix solution A and solution B
8. Fehling's solutions	Solution A: 34.66 g copper sulphate + distilled water to make final volume 100 ml. Solution B: 173 g potassium sodium tartarate + 50 g NaOH + distilled water to make 100 ml
9. Millon's reagent	1 g mercury + 9 ml fuming nitric acid + equal amount of distilled water (after completion of reaction)

Table 2: Protocols for the determination of the alkaloids, amino acids, glucosides, phenolic compounds, steroids and triterpenoids

	Alkaloids
Dragendorff's test	1 ml HCT aqueous extract + 1 ml of dragendorff'sreagent and stir for few min.
Mayer's test	1 ml HCT aqueous extract +1 ml of mayer's reagent and stir for few min.
Wagner's test	1 ml HCT aqueous extract + 1 ml of wagner's reagent and stir for few min.
Hager's test	1 ml HCT aqueous extract + 1 ml of hager's reagent and stir for few min.
Tannic acid test	1 ml HCT aqueous extract + 1 ml of tannic acid solution and stir for few min.
Amino acids	
Millon's test	1 ml HCT aqueous extract + 2 ml of millon's reagent and stir for few min.
Ninhydrin test	1 ml HCT extract + 2 ml of ninhydrin solution and stir for few min.

Anthraquinone glycosides	
Borntrager's test	Boil the 5 ml HCT extract and add 1 ml of $H_2SO_4$and keep aside for 5 min followed by filtration and add equal amount of dichloromethane and separate the upper layer and add half of its volume of ammonia.
Modified Borntrager's test	Boil the 5 ml HCT extract and add 2 ml of H_2SO_4 and 2 ml of 5% aqueous ferric chloride solution and stir for 5 min and shake and add equal amount of dichloromethane and separate the upper layer, add half of its volume of ammonia.
Cardiac glycosides	
Kedde's test	5 ml of HCT extract + one drop of 90% ethanol + 2 drops of 2% 3, 5-dinitro benzoic acid prepared in 90% alcohol. Adjust the pH (8.0 to 9.0) with NaOH solution.
Killer-killani test	5 ml of HCT extract + glacial acetic acid (0.4 ml) + 1 drop of ferric chloride + concentrated H_2SO_4 (0.5 ml).
Raymond's test	2 ml of HCT extract + hot methanolic alkali.
Saponin glycosides	
Froth formation test	2 ml of HCT extract in test tube and shaken vigorously and observation for stable froth (foam).
Phenolic compounds (Tannins)	
Ferric chloride test	2 ml of HCT extract in test tube + ferric chloride solution (1 ml).
Phenazone test	2 ml of HCT extract in test tube + 0.5 g of sodium acid phosphate and warm and filter. To the filtrate add 2% phenazone solution.
Gelatin test	2 ml of HCT extract in test tube + 1% gelatin solution containing 10% NaCl.
Steroids and triterpenoids	
Libermann-Burchard test	2 ml of HCT extract in test tube + few drops of acetic anhydride and boil and cool add concentrated $H_2SO_4$from the walls of the test tube.
Salkowski test	2 ml of HCT extract in test tube + few drops of concentrated H_2SO_4 from the walls of the test tube.
Sulphur powder test	2 ml of HCT extract in test tube + sulphur powder. Observe for shrinking at bottom.

2.7 UV spectroscopic analysis

In UV-Visible spectroscopy, amount of UV or visible light are measured as they are absorbed or transmitted by a sample, relative to a blank reference sample (Shinde *et al.*, 2020). Depending on the sample composition, this feature may reveal what is present in the sample and at what concentration (Siligardi *et al.*, 2014). UV-visible spectroscopic analysis of HCT extract was carried out using calibrated UV-1900 (Shimadzu, Japan) spectrophotometer at spectrum mode. Samples were properly diluted before the evaluation and set to autocorrect before and after every sample analyzed.

2.8 Fourier transform infrared spectroscopy (FTIR) analysis

It is possible to obtain an infrared spectrum of solid, liquid, or gas absorption or emission by using the fourier transform infrared spectroscopy (FTIR) method (Kazarian and Ewing, 2013). As part of this study, FTIR spectrophotometer is used to acquire band intensity in a wide range of wavelengths. There is a substantial advantage to this method over dispersive spectrometers, which measure intensity across a limited range of wavelengths at a time (Blum and John, 2012). FTIR was performed to characterize HCT extract using FTIR (8400S, Shimadzu, Japan) for absorbance vs wavenumber. Samples were properly diluted before the evaluation and peaks were observed between 200-4000 cm^{-1}.

2.9 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

MTT tests assess cell viability, proliferation, and cytotoxicity by measuring cellular metabolic activity (Bahuguna *et al.*, 2017). In this colorimetric test, purple formazan crystals are transformed into yellow tetrazolium salts (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) by metabolically active cells (Morgan, 1998). For cell viability measurement, MDAMB231 cells seeded in 96 well-plates at a cell density of $5x10^3$ and incubated with different concentration of HCT extract such as 220 µg/ml, 240 µg/ml and 260 µg/ml. MTT assay was then carried out as per the instructions written on the manufacturer's guideline. Briefly, 10 µl of MTT reagent was supplemented to each group inside well, followed by additional 4 h incubation. After 4 h, each plate was measured for intensity using multimode UV-microplate reader at 450 nm. Following formula was used to calculate the quantitative % cell viability.

$$\% \text{ Cell viability} = \frac{AB_{sample} - AB_{blank}}{AB_{negative\ control} - AB_{blank}} \times 100$$

where AB_{sample}, AB_{blank}, $AB_{negative\ control}$ denoted the absorbance of samples, blank, and negative control, respectively.

2.10 *In vitro* blood compatibility assay

Blood compatibility test are typically performed as a primary screening to determine hemocompatibility and cytotoxicity. It is an indicative of safety of the extract when it interacts with blood components. For hemocompatibility determination, whole mouse blood collected in heparin treated vials was followed by centrifugation at 4000 g for 5 min to separate the RBCs. After washing with saline solution thrice, the final volume settled to get 2 % w/v RBC suspension. To prepare positive and negative controls, 0.5 ml of triton-x-100 (10% w/v) and 0.5-ml saline were added to 4.5 ml RBC suspension, respectively. HCT extract (0.5 ml each) of different concentration (220-260 µg/ml) were added to 4.5 ml of RBC suspension separately and kept aside for 2 h under mild shaking. All three test tubes were then centrifuged at 4000 g for 15 min to get the supernatant. Supernatant from each group was analyzed using a UV spectrophotometer (UV 1900, Shimadzu, Japan) at 540 nm. Calculations were made following formula:

$$\% \text{ Haemolysis} = \frac{\text{As} - \text{Ab}}{\text{Apc} - \text{Ab}} \times 100$$

As, Ab, and Apc stands for absorption of sample, blank and positive control, respectively.

2.11 Statistical analysis

The various data were analyzed using Prism or Origin softwars. The mean ± SD of triplicate measurementswere analyzed using a one-way variance analysis (ANOVA) and the significant difference was set at $p<0.05$.

3. Results

3.1 Collection, drying and authentication of HCT

HCT ariel parts were collected locally from Guwahati, Assam State of India during the flowering session and utilized to get the aqueous extract. Photographs of the plant is shown in Figure 1.The plant was duly authenticated after due submission of a specimen as herbarium.

3.2 Preparation of HCT extract

For extraction, purified water and dried powder were macerated at a ratio of 10:1 for three times for 24 h. For extraction, purified water and dried powder were macerated at a ratio of 10:1 for three times for 24 h. Afterwards, the obtained material was completely dried at room temperature for 36 h. The dried material was then subjected to lyophilization to finally obtained fine powder as shown in Figure 2.

Figure 1: Photographs of HCT plant (Guwahati, Assam, India).

Figure 2:Completely dried aqueous HCT extract after lyophilization.

3.3 Determination of % yield of HCT extract

After the preparation of HCT extract, % yield was calculated using following formula:

$$\% \text{ Yield of extract} = \frac{\text{weigth of dried extract (g)}}{\text{wight of dried plant (g)}} \times 100$$

$$\% \text{ Yield of extract} = \frac{318(\text{g})}{3000(\text{g})} \times 100 = 10.6\%$$

Percentage yield of HCT extract was found to be 10.6 %. The completely dried aqueous HCT extract after lyophilization is shown in Figure 2 and stored in refrigerator for further investigations.

3.4 Phytochemical investigation of HCT extract

The HCT extract obtained was subjected to various qualitative chemical tests to determine the presence of various phytoconstituents as presented in Table 3. The results of the various tests performed are shown in Table 3.

Table 3: Results of various tests performed

Test	Result	Inference (Pass or fail)
Alkaloids		
Dragendorff's test	Reddish-brown precipitate was observed	Pass
Mayer's test	Cream-colored precipitate was observed	Pass
Wagner's test	Reddish-brown precipitate was observed	Pass
Hager's test	Yellow precipitate was observed	Pass
Tannic acid test	Buff coloured precipitate was observed	Pass
Amino acids		
Millon's test	White precipitate was observed	Pass
Ninhydrin test	Violet colour was observed	Pass
Anthraquinone glycosides		
Borntrager's test	A rose pink to red colour was observed in the ammonical layer	Pass
Modified Borntrager's test	Oxidation was observed	Pass
Cardiac glycosides		
Kedde's test	No colour observed	Fail
Killer-killani test	Bluish-green colour was observed	Pass
Raymond's test	No colour change observed	Fail
	Saponin glycosides	
Froth formation test	Formation of stable froth (foam) observed	Pass
	Phenolic compounds (Tannins)	
Ferric chloride test	Green colour observed	Pass
Phenazone test	No precipitation observed	Fail
Gelatin test	Precipitate observed on the bottom of the test tube	Pass
Steroids and triterpenoids		
Libermann-Burchard test	Deep red colour observed	Pass
Salkowski test	Yellow coloured lower layer observed	Pass
Sulphur powder test	Sinking at the bottom was observed	Pass

3.5 UV-Visible spectroscopic and FTIR characterization

UV-Visible spectroscopy was employed to characterize the different formulations formed at different stages of formulation. The peak at 275 nm as shown in Figure 3A was the characteristic of HCT extract and indicated successful extraction. Similarly, the FTIR spectroscopy was employed to characterize the HCT extract based upon peaks obtained. The band shown at 1743.65 cm^{-1} and 1508.33 cm^{-1} in Figure 3B were characteristic of HCT extract.

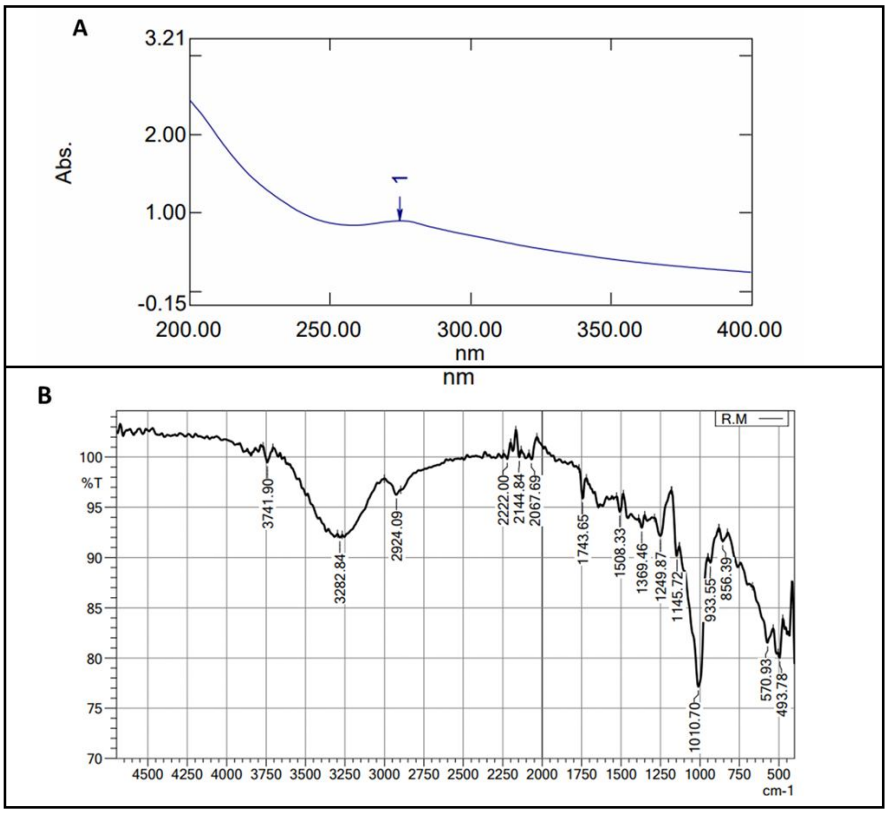

Figure 3: A. UV-visible spectroscopy and B. FTIR analysis of the HCT extract.

3.6 Anticancer activity on MDAMB231 cells

MDAMB231 cancer cell line was chosen to evaluate the *in vitro* combined anticancer potential of different concentration of HCT extract in a dose-dependent fashion. The different concentration used were 220 µg/ml, 240 µg/ml and 260 µg/ml. As compared to 220 µg/ml (~35 % decrease in viability), 240 (~57 % decrease in viability), and 260 µg/ml (~85 % decrease in viability) showed better killing efficiency. In particular, 260 µg/ml extract concentration showed maximum decrease in the viable cell counts as a result of increased concentration (Figure 4).

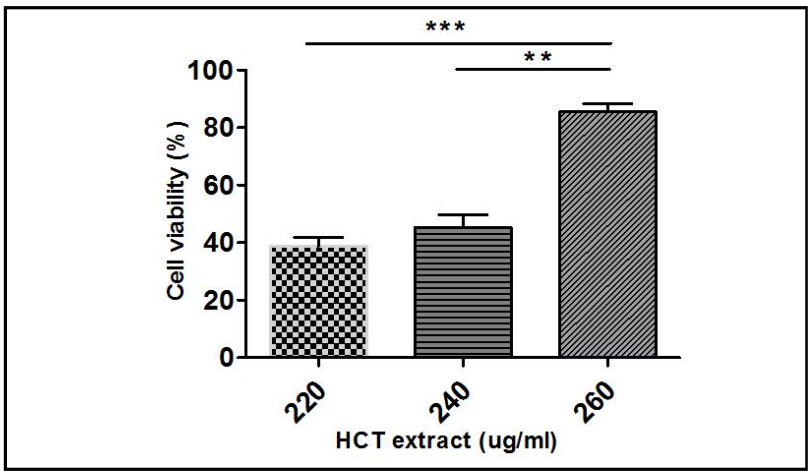

Figure 4: MTT assay analysis depicting the dose-dependent cell viability of HCT extract on MDAMB231 cells.

3.7 *In vitro* hemocompatibility assay

It is fact that the properties of several materials at bulk level and nanoscale differs significantly from each other. Prediction of nanoscale material's interaction with blood and blood components is particularly important for parenteral administration. Although, the HCT extract composed of well-tolerated constituents and biocompatible material, we evaluate the compatibity of different concentration of HCT extract with blood. The results as presented in Figure 5, clearly signifies no issues with the hemocompatibility of all the tested concentration of HCT extract, except the positive control (p>0.05).

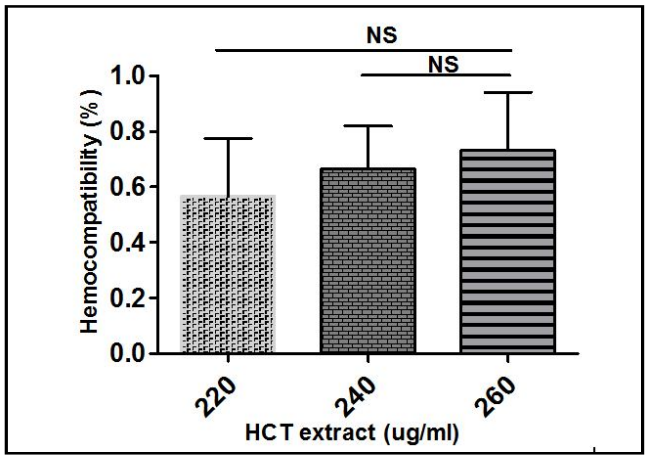

Figure 5: % Hemocompatibility assay of HCT extract at different concentration.

4. Discussion

HCT arial parts were successfully collected, authenticated, dried to get an aqueous extract. After getting the extract, it was again lyophilized to obtained the dry powder. The obtained dry powder was then used to carry out the further investigation. % yield of HCT can be considered appropriate and an indicative of the successful extraction without a significant loss. The HCT extract was then evaluated to verify the presence of various phytoconstituents as presented in Table 3. The results suggested that the aqueous extract of the HCT contains majority of them. This again implies that the aqueous extraction of HCT can preserve the integrity of the plant constituents. Further, the UV-visible and FTIR spectroscopy revealed the successful formation of extract as the characteristic peaks were visible.

Our findings of anti-TNBC activity revealed that HCT plant extract can be a potential candidate to elicit the anticancer effects. The findings also inveterate that the anticancer effect exerted by HCT extract was dose-dependent and more promising at a dose of 260 µg/ml. Our results suggested that HCT extract could be a possible approach to use against the TNBC and can be serve as a chemo-free treatment strategy. This is particularly important to avoid chemo-associated adverse effects as an approach to attain better patient compliance. Our findings are in good relation with those observed by Lai *et al.* (2010) wherein they tested HCT extract against human primary colorectal cancer cells and revealed that HCT extract can potentially inhibits cell growth and induces apoptosis.

In case of hemocompatibility assay, non-significant interaction of the HCT extract (less than 0.8%) with blood components to produce hemolysis confirmed the high blood compatibity of the selected concentration range of HCT extract. Toxicity has direct relation with safety of the pharmaceutical product. All formulations showed Â1.8% hemolysis, and therefore high hemocompatibility further advocating the safe nature. Our results are in good correlation with the finding of El-Far *et al.* (2018) who documented the non-hemolytic nature of folate functionalized plant bioactive resveratrol loaded phytosomes as no homolysis were observed with all the tested concentrations (100 and 250 µg/ml).

5. Conclusion

The practice of plant-based medicine dates back thousands of years and continues to be practiced today. Some therapies have been passed down from generation-to-generation, while others are more recent inventions. In the present investigation, the anticancer activity of an aqueous extract of HCT arial parts was confirmed in this investigation. The presence of different plant actives was also determined using the standard technique. In conclusion, our results confirmed the that HCT extract can induce apoptosis in MDAMB231 cells in concentration dependent manner. Thus, the present work using aqueous extracts of HCT arial parts was found to be successful in treating TNBC. As a result of these findings, a new avenue has opened for the application of HCT extract in conjunction with other chemotherapy drugs or alternatively in the context of combination anticancer therapies.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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