Standardization of a polyherbal formulation (HC9) and comparative analysis of its cytotoxic activity with the individual herbs present in the composition in breast cancer cell lines

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ABSTRACT

Introduction: The present study aims to standardize a polyherbal formulation (HC9) that was previously shown to exhibit excellent antioxidant and cytotoxic activity in breast cancer cells. Here, we have compared the cytotoxic activity of HC9 with its individual components in breast cancer and non-cancerous cells. **Methods:** Physico-chemical and phytochemical evaluation of HC9 was performed. Qualitative and quantitative HPTLC analysis of component herbs and HC9 was done by using specific markers. The cytotoxic activity of HC9 with its individual components was evaluated in breast cancer (MCF-7 and MDA MB-231) and non-cancerous cell lines (HEK-293, HaCaT and MCF-10A) by MTT dye uptake. Results: Physico-chemical results revealed that HC9 contained 7.24% total ash content, 9.52% of alcohol-soluble extractive, 0.801 specific gravity, 0.50g/ml bulk density and exhibited 7.18% loss on drying. Phytochemical results revealed the presence of alkaloids, carbohydrates, flavanoids, saponins, tannins and phenolic compounds, and absence of terpenoids. The individual herbs of HC9 and the formulation showed the presence of marker compounds such as picroside-I, nootkatone, 6-gingerol, matairesinol, swertiamarin, berberine, connesine and 2-hydroxy-4-methoxybenzaldehyde. At 160μg/ml concentration, HC9 exhibited cytotoxicity in both MCF7 and MDA MB231 with no cytotoxicity in MCF-10A, HaCaT and HEK-293. In contrast, at this concentration, the individual herbs of HC9 exhibited cytotoxicity not only in cancerous cells, but also in non-cancerous cells. **Conclusion:** These results suggest that the standardized HC9 formulation was safe to non-cancerous cells and exhibited significant antineoplastic potential in breast cancer cells. Thus, HC9 could be a potential drug candidate in breast cancer.

Keywords: Cytotoxicity, HPTLC, physicochemical, polyherbal formulation HC9, phytochemical, standardization.

INTRODUCTION

Herbal medicines have gained global importance over the past few decades.[1–6] Their medicinal and economic benefits have been accepted in both developing and industrialized nations, particularly in developing countries, where they are being traditionally used against various disease complications.[7–9] Herbal remedies, in the form of a single herb or polyherbal formulations, play a prime role in the healthcare system because of their wide biological activity, easy accessibility, cost effectiveness and safe usage.[10–12] However, these medicines have not yet integrated into the modern clinical practice due to lack of experimental and clinical evidence on their quality.[13,14]

The complexity of polyherbal formulations impose a greater challenge in establishment of their quality, efficacy and safety, compared to single herbal counterparts.[15–17] Thus, it becomes important to standardize the herbal drugs by various parameters and sophisticated techniques to ensure their quality, safety and efficacy.[18–20] Various

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regulatory bodies such as World Health Organization (WHO), European Agency for the Evaluation of Medicinal Products (EMEA), United States Pharmacopeia (USP), and Department of AYUSH, Government of India, have provided the standardization guidelines for development of herbal preparations.[21,22]

In the present work, we have standardized a polyherbal formulation (HC9) that we previously reported to possess antioxidant and cytotoxic activity.[1] It is composed of nine medicinal herbs that include *Picrorhiza kurroa*, *Cyperus rotundus, Zingiber officinale*, *Cedrus deodara*, *Tinospora cordifolia*, *Holarrhena antidysenterica*, *Swertia chirata*, *Cissampelos pareira* and *Hemidesmus indicus* (Table 1). The formulation was prepared based on the reported anticancer and immunomodulatory activity of the individual herbs present in it (Table 2). We have standardized each herbal component of HC9 and the composite formulation with respect to their marker compounds. We have also evaluated the physicochemical and phytochemical parameters of HC9. After standardizing HC9, we have compared the cytotoxic activity of HC9 with its individual components in human breast cancer (MCF-7 and MDA MB-231) and non-cancerous transformed (HEK-293 and HaCaT) cell lines. This was done to evaluate whether the individual components of HC9 were more active than the whole formulation.

MATERIALS AND METHODS

Chemicals and reagents

Tissue culture plasticware was purchased from BD Biosciences (CA, USA) and Axygen Scientific Inc (CA, USA). Dulbecco's Modified Eagles Medium (DMEM) powder, penicillin and streptomycin were obtained from Invitrogen/Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). All other common solvents were procured from Qualigen Fine Chemicals (Mumbai, India) and HPTLC grade solvents were purchased from Merck (Mumbai, India). Reference marker compounds for High Performance Thin Layer Chromatography (HPTLC) analysis were obtained from the Natural Remedies Pvt Ltd (Bangalore, Karnataka-India) and Sigma-Aldrich (St. Louis, MO, USA). The solvents used for high-performance thin-layer chromatography (HPTLC) analysis were obtained from MERCK (Mumbai, India).

Collection, identification and authentication of plant materials

The whole/parts of all nine component herbs of HC9 were purchased from Shri Shailya Medi-Pharms (Solapur, Maharashtra, India). The individual bulk herb samples were stored in air-tight containers and kept in air-conditioned environment until further use. The samples were authenticated and validated macroscopically and microscopically in Department of Botany, Agharkar Research Institute (ARI), Pune (Maharashtra, India). Voucher specimens of herbs have been deposited at the Department of Botany, Agharkar Research Institute and Herbaria of Medicinal Plant Conservation Centre (MPCC), Pune (Table 1).

Extract preparation

All nine herbs of HC9 were washed, dried and fine powdered separately. Ethanolic extracts of individual herbs were prepared by soxhlet extraction method.[1] For the preparation of HC9 formulation, equal parts of each powdered plant material of HC9 were mixed in 1:1 ratio and subjected to soxhlet extraction method using ethanol. The resulting extracts were centrifuged at 13000rpm for 15 min to remove the particulate matter. The supernatants were filter-sterilized using Swiney filter (pore size, 0.45 μm) and the resultant filtrates were stored in aliquots at -80°C until further use.

Organoleptic evaluation of HC9

The organoleptic characters of the powdered HC9 were evaluated by appearance, size, shape, color, texture, odor and taste according to the guidelines of Indian Pharmacopoeia.[47]

Table 1. Composition of polyherbal formulation (HC9).

Plant materials in HC9	Family	Parts used	Voucher specimen no.
Picrorhiza kurroa	Plantaginaceae	Root	R-120
Cyperus rotundus	Cyperaceae	Rhizome	R-121
Zingiber officinale	Zingiberaceae	Bark	R-122
Cedrus deodara	Pinaceae	Root	S/B-096
Tinospora cordifolia	Menispermaceae	Stem	S/B-097
Holarrhena antidysenterica	Apocynaceae	Seed	S-119
Swertia chirata	Gentianaceae	Whole plant	WP-078
Cissampelos pareira	Menispermaceae	Root	MPCC 290
Hemidesmus indicus	Apocynaceae	Root	MPCC 2354

This table shows nine plant materials, their families, voucher specimen numbers alongwith the parts used in the preparation of polyherbal formulation (HC9).

Table 2. Properties of individual herbs of HC9.

Herbs in HC9	Reported anticancer activity	Medicinal properties	References
Picrorhiza kurroa	Breast and skin cancer Protect against Adrynomycin induced cardomyopathy	Antioxidant, immunomodulatory, antibacterial, antiperiodic, hepatoprotective, antiasthamatic, gastrointestinal, anti-urinary activity	23–26
Cyperus rotundus	Gastric cancer, lymphoma, leukemia, cytotoxic and apoptotic role	Appetizer, antioxidant, immunomodulatory, anti-inflammatory, antidiabetic, antidiarrhoeal, cytoprotective, antimutagenic, antimicrobial, high estrogens reducer, breast pain inhibitory activity	27–29
Zingiber officinale	Breast, cervical, colon, lung, ovary, prostate cancer protect against Doxorubicin induced acute nephrotoxicity	Antioxidant, appetizer, anti-inflammatory, antiangiogenic, cardiotonic, antiplatelet, antihepatotoxic, antifungal activity	30–32
Cedrus deodara	Breast, cervical, skin, leukemia, colon, lung and ovary cancer	Anti-inflammatory, immunomodulator, anti-ulcer, anti-fungal, anti-arthritic, anti-allergic, anti-oxidant activity	33–35
Tinospora cordifolia	Prostate, liver, skin and breast cancer Protect against chemo induced leucopenia	Immunomodulator, antioxidant, anti-inflammatory, anti-stress, gastrointestinal and hepatoprotection, anti-allergic activity	36–38
Holarrhena antidysenterica	Anticancer	Appetizer, anti-diabetic, diarrhea, anti-oxidant activity	39
Swertia chirata	Skin cancer	Blood purifier, appetizer, Antioxidant, , anti-inflammatory, immunomodulator, anti-hepatotoxic, antidiabetic, antimicrobial activity	40–41
Cissampelos pareira	Lung, leukemia, lymphoma	Antioxidant, , immunomodulator, anti-inflamatory activity, skin diseases, gastric ulcers, cardiac and abdominal pain reducer	42–43
Hemidesmus indicus	Hepatocancer	Blood purifier, appetizer, antioxidant , anti-inflammatory, anti-microbial, anti-hepatotoxic activity and used to treat kidney, urinary and skin diseases	44–46

This table shows the reported anticancer, immunomodulatory as well as other medicinal properties of component herbs of HC9.

Determination of physicochemical parameters of HC9

Physico-chemical parameters such as total ash content, total viable count, loss on drying of extract, ethanol-extractable matter in the mixture of air-dried powder material, determination of pH, bulk density as well as specific gravity of HC9 extract were carried out at Indian Drug Research Institute (IDRI), Pune (Maharashtra, India) according to the prescribed standard methods in Indian Pharmacopoeia.[47]

Preliminary phytochemical analysis of HC9

The preliminary phytochemical analysis of HC9 was done by Indian Drug Research Institute (IDRI), Pune (Maharashtra, India). The extract was screened to detect the presence of secondary metabolites such as alkaloids, carbohydrates, flavanoids, saponins, terpenoids, tannins and phenolic compounds.[47]

HPTLC finger printing profile

Identity of individual herbs and HC9 formulation was confirmed by detecting the presence of marker compounds such as picroside-I, nootkatone, 6-gingerol, matairesinol, berberine, connesine, swertiamarin, berberine and 2-hydroxy-4-methoxybenzaldehyde in *P. kurroa*, *C. rotundus, Z.officinale*, *C. deodara*, *T. cordifolia*, *H. antidysenterica*, *S. chirata*, *C. pareira* and *H. indicus,* respectively. Stocks and working solutions of different marker compounds were prepared in respective diluents (Table 3). Standard (marker compounds) and samples (HC9 and nine individual herbs) were applied onto a thin layer chromatography (TLC) plate, using an automatic TLC sampler (Linomat 5) as described previously.[5]

In brief, the samples (standards and test samples) were spotted as bands (8mm width) with a Camag (Muttenz, Switzerland) Hemilton microlitre syringe onto a pre-coated aluminum-backed silica gel 60F-254 plate (20 × 10cm; layer thickness 250µm; Merck, Darmstadt, Germany) using a Camag high-performance thin-layer chromatography (HPTLC) system equipped with an automatic TLC sampler (Linomat 5), TLC scanner 3, and integrated software Win-Cats version 4. A constant application rate (0.1µL/s) was employed and the space between the two bands was 6 mm. The respective working solutions of standards (Table 3) were applied to the TLC plate along with the test solution. Linear ascending development was carried out in 20cm × 10cm twin trough glass

Table 3. Solvents of marker compounds along with their concentrations.

Marker compounds	Solvents	Stock solution (mg/ml)	Working solution (mg/ml)
Picroside-I	Methanol	2	0.2
Nootkatone	Methanol	10	0.1
6-Gingerol	Methanol	10	2
Matairesinol	Methanol	1	0.1
Berberine	Methanol	2	0.02
Conesine	Methanol	1	1
Swertiamarin	Methanol	2.3	0.02
2-hydroxy-4-methoxybenzaldehyde	n-Hexane	10	0.2

This table shows respective marker compounds of individual herbs of HC9 along with their diluents, stock and working solutions.

chamber pre-saturated with the respective mobile phase. The optimum chamber saturation time for the mobile phase was 20 min at room temperature. The chromatoplates were developed up to 80mm under chambersaturation conditions to get good resolution of phytochemical contents. Subsequent to development, TLC plates were dried in a current of air with the help of an air-dryer to evaporate solvents from the plates. The plates were examined using A Camag model III TLC scanner with CATS 4.0 integration software. Densitometric scanning was performed in the appropriate absorbance mode with a slit dimension of 6 × 0.45mm and ascanning speed of 10mm/s. A deuterium lamp was used as source of radiation. The amount of marker compounds present in HC9 was determined from the calibration curve obtained by plotting the concentration of standard against the peak area of test samples.

Cell lines

The human breast carcinoma cell lines, MCF-7 and MDA MB-231 and non-cancerous transformed cell lines, HEK-293 (Human Embryonic kidney) and HaCaT (Human Keratinocyte) used in the study were obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in DMEM containing 2mM L-glutamine supplemented with 10% fetal bovine serum and 100U/ml of penicillin-streptomycin. The cells were incubated in a humidified 5% CO2 incubator at 37°C. Non-tumorigenic normal mammary epithelial cell lineMCF-10A was a kind gift from Dr. Milind Vaidya (ACTREC, Mumbai). The cells were grown in DMEM:Ham's F12 (1:1) containing 2 mM L-glutamine supplemented with 10% fetal bovine serum, 100U/ml of penicillin-streptomycin, 10µg/ml insulin, 20ng/ml EGF and 0.5 mg/ml hydrocortisone. The cells were incubated in a humidified 5% CO2 incubator at 37°C.[48]

Cytotoxic Assay

Cytotoxicity of nine component herbs of HC9 and the whole formulation was determined in the cancerous and non-cancerous cell lines by MTT dye uptake.[49] Briefly, MCF-7, MDA-MB-231, HEK-293 HaCaT and MCF-10A cells were seeded at 1 × 10^5/ml density in 96-well plates. Next day, the cells were incubated with various concentrations of HC9 and ethanolic extracts of individual herbs (0–160µg/ml) for 24 h and incubated in 5% CO2 incubator at 37°C. Next day, the MTT solution (5mg/ml) was added to each well and the cells were cultured for another 4 h at 37°C in 5% CO2 incubator. The formazan crystals formed were dissolved by addition of 90µl of SDS-DMF (20% SDS in 50% DMF). After 15 min, the amount of colored formazan derivative was determined by measuring optical density (OD) with the ELISA microplate reader (Biorad, Hercules, CA) at OD 570–630nm. The percentage viability was calculated as:

% Viability = [OD of treated cells/ OD of control cells] × 100

Statistical analysis

IC_{50} values were calculated by using Kyplot software. All the assays were performed in triplicates and repeated at least three times at different time points. The data has been presented as IC_{50} values and mean ± SD.

RESULTS AND DISCUSSION

Organoleptic evaluation

The powdered HC9 was evaluated for its organoleptic properties. The results revealed that HC9 was dark green in color with characteristic odor, bitter taste and fine texture. These parameters form the basic criteria for selecting a raw drug.[54] Fine texture of powdered HC9 indicated the smoothness and surface uniformity that forms the primary character to assess the quality of a herbal drug.[50]

Physico-chemical analysis

HC9 was evaluated for total ash content, ethanol soluble extractive, loss on drying at 105°C, pH, specific gravity and bulk density. All the values have been summarized in Table 4a. Physico-chemical analysis of HC9 revealed that the total ash content present in HC9 was 7.24%. The total

Table 4 (a) Physio-chemical characteristics of HC9.

Parameters	Values
Total ash content	7.24%
Ethanol extractives	9.52%
Loss on drying	7.18%
pH	6.1 ± 0.2
Bulk density	0.50g/ml
Specific gravity	0.801

Table 4(b) Permissible limits of physico-chemical parameters of individual herbs in HC9.

Plant materials in HC9	Total ash content (%)[a]	Ethanol extractives (%)[b]	Loss on drying (%)	References
Picrorhiza kurroa	NMT 7	NLT 10	NMT 13	Ayurvedic Pharmacopoeia of India; Volumes I,III and IV
Cyperus rotundus	NMT 8	NLT 5	–	
Zingiber officinale	NMT 6	NLT 4.5	NMT 7.13	
Cedrus deodara	NMT 2	NLT 7	–	
Tinospora cordifolia	NMT 7	NLT 6	NMT 7.5	
Holarrhena antidysenterica	NMT 7	NLT 18	–	
Swertia chirata	NMT 6	NLT 10	–	
Cissampelos pareira	NMT 7	NLT 11	–	
Hemidesmus indicus	NMT 4.3	NLT 15	–	

[a]NMT: not more than; [b]NLT: not less than, –: not available

This table shows shows permissible limits of physico-chemical parameters of individual herbs in HC9 according to Ayurvedic pharmacopoeia of India; Volume I, III and IV

ash values of the individual plant materials of HC9 have been reported to be in the range of 2–8% (Table 4b). Determination of total ash value is an important criteria to judge the authenticity and purity of the crude drug.[50] It indicates total amount of inorganic material present in the drug after its complete incineration. A high ash value indicates contamination, substitution, adulteration during the preparation of drug.[51] The results indicated that HC9 has low inorganic material.

The percentage yield of alcohol-soluble extractive of HC9 was found to be 9.52% w/w. The alcohol-soluble extractive values of the individual plant materials in HC9 have been reported to be in the range of 4.5–18% (Table 4b). The extractive value indicates the amount of active ingredient present in the given amount of plant material when extracted with respective solvent.[50] Less extractive value indicates addition of exhausted material, adulteration or incorrect processing during drying, storage or formulation preparation. The alcohol-soluble extractive of HC9 was found to be within the acceptable range.

Loss on drying at 105ºC of HC9 was found to be 7.18%. This value is indicative of amount of moisture content present in the drug.[51] The test for loss on drying actually determines water as well as volatile matter content in drug when subjected to heat. The high moisture content in herbal drugs endorses microbial as well as insect contamination. The low moisture content is always desirable for higher stability of drugs. Our results showed that the formulation could be stored for a long period and would not be easily contaminated with microbes.[51]

The pH conventionally represents the acidity or alkalinity. HC9 (1% w/v solution) showed a pH of 6.1 indicating that the formulation was acidic in nature.

Bulk density, a measure used to describe packing of particles or granules, of HC9 was found to be around 0.50 g/ml. Lower value of density indicates good flow and higher value indicates poor flow properties of formulation.[50] The specific gravity of HC9 was found to be 0.801. All these values indicated that HC9 exhibited good flow properties.

Preliminary phytochemical evaluation

The preliminary phytochemical screening of HC9 demonstrated the presence of alkaloids, carbohydrates, flavanoids, saponins, tannins and phenolic compounds, and absence of terpenoids. These qualitative tests are used to detect the presence of functional groups, which play an important role in the biological activity of the drug.[49]

HPTLC analysis of ethanolic extracts of individual components of HC9 and the composite formulation

TLC fingerprinting profile followed by HPTLC analysis of component herb present in HC9 and the formulation was performed by using respective marker compounds

Table 5. Major chemical compounds present in HC9 along with the markers used in the study.

Plant materials	Major chemical compounds	Selected marker compounds in the study	References
Picrorhiza kurroa	Picroside I, Picroside II, Picroside IV and 6-ferulloylcatalpol	Picroside-I	23–24, 26
Cyperus rotundus	α-copaene , cyperene, β-selinene, β-cyperone, nootkatone,valerenal, caryophyllene oxide, α-selinene	Nootkatone	27, 52
Zingiber officinale	6-gingerol, 8-gingerol 10-gingerol and, 6-shogaol	6-Gingerol	30–31
Cedrus deodara	Wikstromol, matairesinol, dibenzylbutyrolactol	Matairesinol	33–34
Tinospora cordifolia	Cordifolioside A, tinocordifolin, berberine, tinosporadine, tinocordifolioside, makisterone,cordifol	Berberine	53–54
Holarrhena antidysenterica	Conesine, antidysentericine	Conesine	39,55
Swertia chirata	Mangiferin, swertiamarin, sweroside,amarogentin, Swertinin, swertianin, swerchirin	Swertiamarin	40, 56–57
Cissampelos pareira	Mensmine, pareirine, hayatinine, bebeerine, beberine, tetrandrine	Berberine	42, 58–59
Hemidesmus indicus	2-hydroxy-4methoxy-benzaldehyde, Hemidesmin 1 and 2, α-amyrin, β-amyrin, lupeol	2-hydroxy-4-methoxybenzaldehyde	44–46

This table shows major chemical compounds present in individual herbs of HC9 along with the markers used in the study for HPTLC analysis of HC9.

Table 6. HPTLC analysis of HC9 and individual plant materials.

Marker compounds	Mobile phase	λ_{max} (nm)	Rf Value	Amount (%) of marker compound in HC9	Amount (%) of marker compound in individual herbs
Picroside-I	Choloform: ethanol [8.8:1.2]	282	0.22	18.41	27.76
Nootkatone	N-hexane: EtOAc [3:7]	249	0.97	4.59	6.59
6-Gingerol	N-hexane: EtOAc [6:4]	282	0.6	12.04	46.03
Matairesinol	EtOAc:MeOH:FA:H2O [7:1.5:0.5:1]	284	0.83	8.89	30.01
Berberine	n-but: EtOAc: GAA:H2O [3:5:1:1]	350	0.29	4.06	10.36
Conesine	Toulene: EtOAc: diethylamine [6.5:2.5:1]	520	0.68	1.66	6.58
Swertiamarin	EtOAc:MeOH:H2O [7.5:1.5:1.2]	244	0.66	4.41	12.43
Berberine	n-but: EtOAc: GAA: H2O [3:5:1:1]	350	0.29	4.2	5.95
2-hydroxy-4-methoxybenzaldehyde	Toulene: EtOAc: GAA [7:2:1]	282	0.91	12.72	13.78

EtOAc: ethyl acetate; MeOH: methanol; FA: formic acid; H2O: water; n-but: n-butanol; GAA: glacial acetic acid
This table summarizes the marker compounds, mobile phases, wavelength (λ_{max}) as well as Rf values of spots visible in the HPTLC profiles of the each herb.

(Table 5). The finger printing profiles were developed in respective solvent systems as given in Table 3 and their corresponding peaks were recorded at respective Rf values. Table 6 summarizes the marker compounds, mobile phases, wavelength (λ_{max}) and R_f values of spots visible in the HPTLC profiles of each herb.

The individual plant extracts showed Rf values of 0.22, 0.97, 0.6, 0.83, 0.29, 0.68, 0.66 and 0.91 corresponding to their marker compounds picroside-I, nootkatone, 6-gingerol, matairesinol, berberine, connesine, swertiamarin and 2-hydroxy-4-methoxybenzaldehyde in the extracts (Table 6). Similarly, HC9 showed Rf values corresponding to the presence of respective marker compounds in the formulation. Thus, all the component herbs of HC9 were authenticated and found to be present in the the formulation based on HPTLC analysis.

The amount of marker compounds present in the extracts of individual plants of HC9 and the formulation was also evaluated. *P. kurroa*, *C. rotundus, Z. officinale*, *C. deodara*, *T. cordifolia*, *H. antidysenterica*, *S. chirata*, *C. pareira*

and *H. indicus* was found to have 27.76, 6.59, 46.03, 30.01, 10.36, 6.58, 12.43, 5.95 and 13.78% of picroside-I, nootkatone, 6-gingerol, matairesinol, berberine, connesine, swertiamarin, berberine and 2-hydroxy-4-methoxybenzaldehyde, respectively (Table 6). on the other hand, HC9 was found to have 18.41, 4.59, 12.04, 8.89, 4.06, 1.66, 4.41, 4.2 and 12.72% w/w of picroside-I, nootkatone, 6-gingerol, matairesinol, berberine, connesine, swertiamarin, berberine and 2-hydroxy-4-methoxybenzaldehyde, respectively (Table 6). The overall results indicated that HC9 contained more amount of picroside-I, 6-gingerol, matairesinol and 2-hydroxy-4-methoxybenzaldehyde compared to the other marker compounds. Thus, the activity of HC9 could be due to the presence of high amounts of picroside-I, 2-hydroxy-4-methoxybenzaldehyde, 6-gingerol and matairesinol.

Effect of individual extracts and HC9 formulation on cell viability

After standardization of HC9, we wanted to compare its cytotoxic activity with the component herbs to know whether the individual components of HC9 were more active than the whole formulation. Thus, cytotoxicity was evaluated in human breast cancer cell lines, MCF-7 and MDA MB-231 as well as in non-cancerous cell lines, MCF-10A, HaCaT and HEK-293.

The cytotoxicity results showed that IC_{50} value of HC9 in MCF-7 was lower (150.29μgml^{-1}) than individual plant extracts except for *S. chirata* showing IC_{50} value of 109.35μgml^{-1} (Table VII). However, *S. chirata* was cytotoxic to non-cancerous cells at lower concentrations with IC_{50} values of 65.87, 24.63 and 71.10μgml^{-1} in MCF-10A, HaCaT and HEK-293, respectively (Table 7).

In MDA MB-231, IC_{50} value of HC9 was lower (184.50 μgml^{-1}) than individual plant extracts except for *Z. officinale, C. deodara,* and *H. indicus* showing IC_{50} values of 176.38, 158.62 and 130.88μgml^{-1}, respectively. However, these herbs were cytotoxic to the non-cancerous cells and showed IC_{50} values of 166.67, 84.33 and 217.23μgml^{-1}, respectively in MCF-10A; 62.36, 58.15, 107.19μgml^{-1}, respectively in HaCaT and 48.71, 50.64 and 105.61, respectively in HEK-293 (Table 7). Interestingly, HC9 showed higher IC_{50} values in MCF-10A (>640μgml^{-1}), HaCaT (>640μgml^{-1}) and HEK-293 (586.10μgml^{-1}) compared to the component herbs (Table 7). HC9 was non-cytotoxic up to 160μg/ml concentration in non-cancerous cell lines and exhibited significant cytotoxicity in MCF-7 and MDA MB-231 at the same concentration (Supplementary data S1–S5). These results suggest that the standardized HC9 formulation was safe to non-cancerous cells and exhibited significant anticancer potential for breast cancer cells compared to the component herbs.

CONCLUSION

Standardization of the polyherbal formulation (HC9) was done according to Ayurvedic Pharmacopoeia of India guidelines (Department of AYUSH, Government of India). The present study may be used as a reference standard for quality control and standardization of polyherbal formulations that could help in strengthening the use of medicinal herbs. Our study suggests that compared to the component herbs, HC9 exhibited significant cytotoxicity in breast cancer cells without killing the non-cancerous cells. Further investigations are underway to identify the underlying mechanisms of antineoplastic activity of HC9 in breast cancer.

Table 7. IC_{50} values of HC9 and individual herbs in breast cancer and non-cancerous cell lines.

Plant materials in HC9	IC50 (μgml^{-1})				
	MCF-10A	HaCaT	HEK-293	MCF-7	MDA MB-231
P.kurroa	351.55	497.61	336.92	320.76	374.60
C. rotundus	193.01	131.42	144.23	180.65	331.64
Z. officinale	166.67	62.36	48.71	186.29	176.38
C. deodara	84.33	58.15	50.64	157.50	158.62
T.cordifolia	102.00	211.78	162.47	384.95	471.15
H. antidysenterica	299.95	457.38	188.82	>640	>640
S. chirata	65.87	24.63	71.10	109.35	233.95
C. pareira	89.07	137.04	72.59	291.84	492.92
H. indicus	217.23	107.19	105.61	182.99	130.88
HC9	>640	>640	586.10	150.29	184.50

This table summarizes the IC_{50} values of HC9 and individual herbs that were dosed at different concentrations (0–640 μg/ml) in non-cancerous and breast cancer cell lines.

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