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ORIGINAL ARTICLE

Tumor Retardation and Immunomodulatory potential of Polyherbal Formulation HC9 in Mouse Melanoma Model

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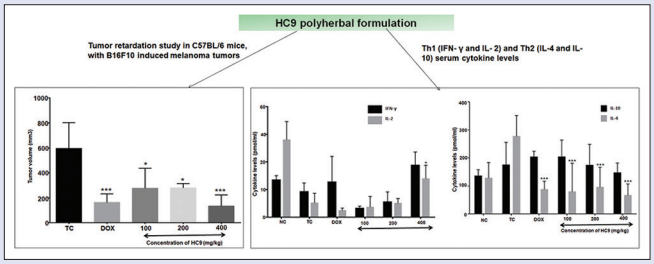
ABSTRACT

Background: HC9, a polyherbal formulation, is based on Stanyashodhana Kashaya (an Ayurvedic formulation) that is being prescribed by Ayurvedic physicians for the treatment of various disorders of mammary glands. We have recently reported anticancer activity of HC9 in breast cancer cell lines through various molecular mechanisms. Few studies have shown an association between breast cancer and melanoma that has prompted us to find whether HC9 could regulate the melanoma growth as well. **Aim of the Study:** The aim was to investigate the tumor retardation and immunomodulatory potential of HC9 in mouse melanoma model. **Materials and Methods:** C57BL/6 mice, with B16F10-induced melanoma tumors, were divided into six groups: tumor control, doxorubicin (2 mg/kg body weight [b.w.]), low dose (100 mg/kg b.w.), intermediate (200 mg/kg b.w.), and high dose (400 mg/kg b.w.) of HC9. No tumor control served as the negative control group. The mice were orally gavaged with HC9 daily for 3 weeks. The urine and blood samples from all the animals were taken before necropsy. The expression of T-helper type 1 (Th1) (interferon-γ and interleukin [IL]-2) and Th2 (IL-4 and IL-10) serum cytokines was evaluated by ELISA assay. **Results:** HC9 significantly retarded the tumor growth in C57BL/6 mouse melanoma model. The animals did not show any changes in body weight and food consumption throughout the study period. Urine and histopathological analysis revealed no signs of toxicity in HC9-treated animals. HC9 appreciably increased the serum levels of Th1 with a concomitant decrease in Th2 cytokines. **Conclusion:** HC9 retarded the tumor growth in mouse melanoma model and induced immunomodulation, thereby suggesting the potential of the formulation against melanomas.

Key words: Anticancer, HC9 formulation, immunomodulation, melanoma model, T-helper type 1 and T-helper type 2 cytokines

SUMMARY

- HC9 retarded tumor growth in C57BL/6 mouse melanoma model
- HC9 increased the serum level of Th1 cytokines and decreased Th2 cytokines
- There was no difference in the body weight and food consumption of animals treated with HC9
- Urine and histopathological analysis revealed no signs of toxicity in HC9-treated animals.



Abbreviations used: HC9: Herbal composition comprised of 9 medicinal plants; TC: Tumor control; Dox: Doxorubicin; NTC: No Tumor Control; IFN-γ: Interferon-gamma; IL: Interleukin; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B-cells; COX-2: Cyclooxygenase-2; MMP: Matrix metalloproteinase; HIF1-α: Hypoxia-inducible factor 1-alpha; VEGF: Vascular endothelial growth factor; SMAR1: Scaffold/Matrix-associated region-1; CDP/Cux: CCAAT displacement protein/cut homeobox.

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INTRODUCTION

Epidemiological studies have suggested a relationship between breast cancer and cutaneous melanoma.[1] Interestingly, carriers of genetic mutations in BRCA2 gene have an increased risk of developing melanoma, whereas those having mutations in *CDKN2A* (melanoma susceptibility) gene have a higher propensity to develop breast cancer. It was reported that in young breast cancer patients, there was a 46% elevated risk of developing a second melanoma. Thus, there seems to be an overlap between the pathways underlying the two types of cancers.

Herbal drugs either as single herbs or polyherbal formulations have been shown to exhibit anticancer activity against various cancers. These drugs exhibit various biological activities and are easily accessible, cost-effective, and safe.[2] However, due to lack of extensive scientific and clinical evidences, herbal drugs have not yet found clinical application.[3] Thus, it becomes essential to ascertain the safety and therapeutic efficacy of herbal medicines, particularly, polyherbal formulations at preclinical level.

HC9 polyherbal formulation has been developed based on Stanya Shodhana Kashaya (SSK). The latter has been prescribed by Ayurvedic practitioners mainly for the breast milk detoxification and lactation-related disorders (Charak Samhita, Chapter 22).[4] The original SSK formulation is composed of ten medicinal plants: *Picrorhiza kurroa* Royle ex Benth., *Cyperus rotundus* L., *Zingiber officinale* Roscoe, *Cedrus deodara* (Roxb. ex D.Don) G.Don, *Tinospora cordifolia* (Willd.) Miers, *Holarrhena antidysenterica* (Roth) Wall. ex A.DC., *Swertia*

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chirata Buch.-Ham. ex Wall., *Cyclea peltata* (Lam.) and *Hemidesmus indicus* (L.) R. Br. ex Schult, and *Marsdenia tenacissima* (Roxb.) Moon. In HC9 formulation, only nine plants have been included because of the non-availability of *M. tenacissima*, which is found only in the Himalayan region. HC9 was previously standardized and found to contain marker compounds such as picroside-I, nootkatone, 6-gingerol, matairesinol, swertiamarin, berberine, connesine, and 2-hydroxy-4-methoxybenzaldehyde.[5] HC9 exhibited antioxidant activity and significantly reduced the viability of MCF-7 and MDA-MB-231.[5] Acute and subacute toxicity studies in Swiss albino have revealed the safety of HC9 up to 2000 mg/kg body weight (b.w.) of mice with no adverse effects.[6] Recently, we have reported that HC-induced cell cycle arrest reduced migration and expression of angiogenic markers in breast cancer cells.[7] It also regulated the expression of chromatin and inflammatory marker proteins. In the present study, we have evaluated the anticancer activity and immunomodulatory potential of HC9 in a mouse melanoma model.

MATERIALS AND METHODS

Plant material

The nine different plant materials of HC9, such as *P. kurroa* (R-120), *C. rotundus* (R-121), *Z. officinale* (R-122), *C. deodara* (S/B-096), *T. cordifolia* (S/B-097), *H. antidysenterica* (S-119), *S. chirata* (WP-078), *Cissampelos pareira* (Medicinal Plant Conservation Centre [MPCC] 290), and *H. indicus* (MPCC 2354), were purchased from Shri Shailya Medi Pharms, Solapur, Maharashtra, India. They were botanically authenticated at the Department of Botany, Agharkar Research Institute and Herbaria of MPCC, Pune.[7-9]

Preparation of ethanolic extract

HC9 formulation was made by mixing equal parts (1:1 ratio) of each plant material and extracted in ethanol by Soxhlet apparatus as described previously.[7-10]

Experimental animals

The study was sanctioned by the Institutional Animal Ethics Committee (CPCSE Reg. No. 258/CPCSE), Bharati Vidyapeeth University, Pune. Female C57BL/6 mice, 6–8 weeks old with an average weight of 18-22g, were procured from the National Institute of Nutrition (Hyderabad, India). The mice were divided into different groups and housed in polypropylene cages at 21°C ± 3°C with relative humidity of 30%–70% and 12:12 h light/dark rhythm. The mice were acclimatized to laboratory conditions and fed with commercial food pellets (Nutrivet, Pune) and water *ad libitum*.

Tumor induction and its assessment

The tumors were raised in mice by subcutaneous injection of 0.2 ml of B16F10 cells (5×10^5 cells/animal) into the right flank region. Tumors were palpable at the 8th day after the injection of cells after which the animals were grouped with $n = 4$/group. Groups I and II were no tumor control (NTC) and tumor control (TC), respectively, which received distilled water. Group III was positive control (PC) which received doxorubicin (Dox) (intravenous [i.v.], 2 mg/kg b.w.) on the 1st, 5th, and 9th days after development of tumors. Groups IV, V, and VI were orally gavaged with low (100 mg/kg b.w.), intermediate (200 mg/kg b.w.), and high (400 mg/kg b.w.) doses of HC9, respectively, daily for 2 weeks. Food consumption, body weights, and tumor sizes were recorded after every 3 days. Tumor volume (mm^3) was calculated as: 0.5 × shortest diameter^2 × largest diameter. Percentage of tumor growth inhibition was calculated as: (Average tumor volume of control group − Average tumor volume of test group)/(Average tumor volume of control group) ×100. The urine and blood samples were collected before necropsy of animals. After necropsy, different organs were collected for histopathological analysis.

Determination of serum cytokine levels

The serum cytokines (interleukin [IL]-2, IL-4, IL-10, and interferon [IFN]-γ) levels were determined using mouse T-helper type 1/T-helper type 2 (Th1/Th2) ELISA-Ready-Set-Go (eBioscience, San Diego, CA, USA) kit after following the manufacturer's instructions.[11] Readings were taken at 450 nm with FLUOstar Omega microplate reader (CA, USA).

Statistical analysis

The data were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). The experiment was done once, and values with $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered to be statistically significant.

RESULTS

HC9 retarded tumor growth

Oral administration of HC9 significantly reduced the tumor growth in the mice. After comparing with the TC group, mice treated with Dox, HC9 100, 200, and 400 showed significant reduction in the tumor volume by ~65.3 ± 28.3% ($P < 0.0001$), 53.2% ± 17.45% ($P < 0.05$), 47.3% ± 23.7% ($P < 0.05$), and 76.5% ± 12.7% ($P < 0.0001$), respectively [Figure 1].

HC9 was safe for the animals

No significant change was observed in the body weight [Table 1] and food intake [Table 2] of animals treated with either Dox or different doses (100, 200, and 400 mg/kg b.w.) of HC9 compared to NTC or TC. No significant difference ($P > 0.05$) in the relative organ weights of the treated mice was observed compared to either NTC or TC mice. The urine analysis revealed no signs of toxicity in the HC9-treated mice [Tables 3a-f].

HC9 modulated T-helper type 1/T-helper type 2 cytokine levels

It was observed that compared to the TC group, at 400 mg/kg dose, HC9 increased IFN-γ and IL-2 levels by ~1.7 ($P > 0.05$; NS) and ~2.7 ($P < 0.05$) folds, respectively [Figure 2a]. At 400 mg/kg dose, HC9 decreased IL-10 and IL-4 levels by ~1.2 ($P > 0.05$; NS) and ~4.4 ($P < 0.0001$) folds, respectively, compared to the TC group [Figure 2b].

Table 1: Body weights (g) of female mice from different groups

Days	Body weights (g)					
	Group I	Group II	Group III	Group IV	Group V	Group VI
0	25.8±4.3	25.3±3.8	25.5±3.9	25.3±1.6	25.3±3.4	25.3±3.1
1	26.0±5.2	26.4±4.4	26.5±3.2	26.3±2.2	26.3±3.8	26.6±3.5
5	27.1±3.5	29.1±2.0	26.5±4.2	27.8±4.0	27.0±4.2	27.2±3.7
7	27.6±3.8	28.6±1.9	26.4±4.1	27.1±4.3	26.6±4.2	27.3±3.1
12	27.2±3.5	28.6±1.7	27.3±3.9	28.2±4.3	26.6±4.2	28.1±3.5
15	27.3±3.5	27.9±2.1	26.9±3.6	26.9±3.6	27.7±4.4	26.4±4.1

Body weights of female mice following treatment with vehicle, Dox and different doses of HC9 have been shown. The values represent change in body weight of mice from 0 to 15 days. Each value represents mean±SD (n=4). SD: Standard deviation; Dox: Doxorubicin

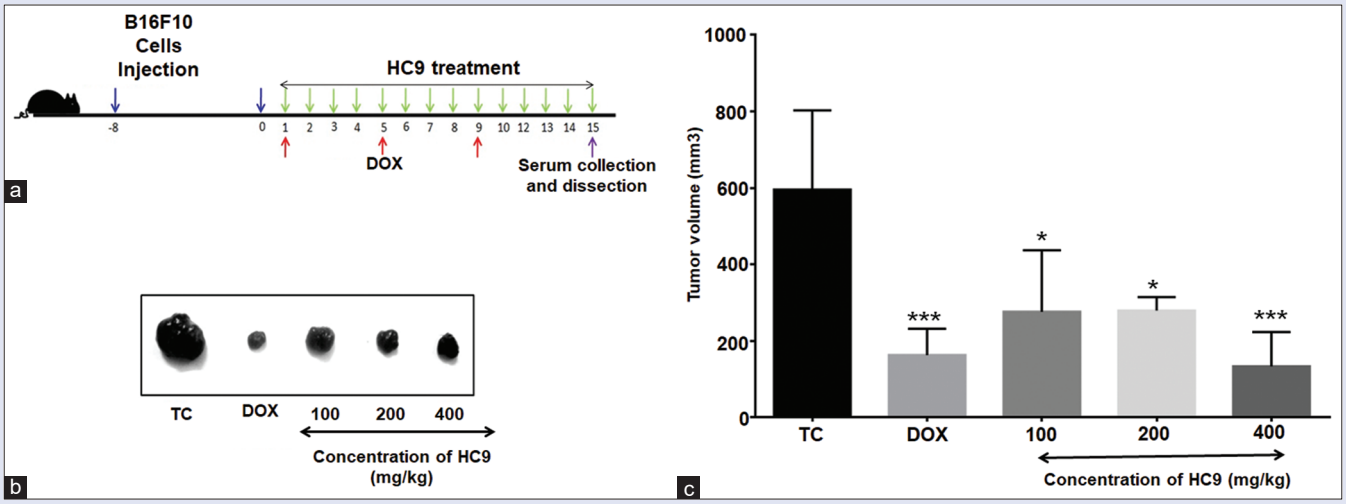

Figure 1: HC9 retarded growth of subcutaneous melanoma tumors in C57BL/6 mice. (a) Scheme showing tumor generation and drug intervention in mice. B16F1 cells were subcutaneously injected at a density of 5 × 105 cells/animal. The palpable tumors were observed on the 8th day. Doxorubicin was given on the 1st, 5th, and 9th days once tumors had developed. HC9 was given from day 1 up to 2 weeks. (b) Representative photographs of tumors from tumor control, doxorubicin, and HC9-treated mice. (c) Graph representing a decrease in tumor volume after HC9 treatment. Data have been represented as mean ± standard deviation. The experiment was done twice (n = 4 mice/group). The values with *P < 0.05 were considered to be statistically significant

Table 2: Food consumption of female mice from different groups

	Food consumption (g)					
	Group I	Group II	Group III	Group IV	Group V	Group VI
Week 1 (1-5)	21.0±0.9	22.8±3.2	19.3±0.9	21.7±4.8	22.0±2.5	20.3±2.6
Week 2 (7-15)	26.0±6.1	27.5±1.7	18.2±9.0	22.3±2.8	26.8±5.0	26.8±8.3

Food consumption of female mice following treatment with vehicle, Dox, and different doses of HC9 has been shown. The values represent change in food consumption of mice at week 1 (1-5 days) and week 2 (7-15 days). Each value represents mean±SD (n=4). Each point represents the mean±SD (n=4). SD: Standard deviation; Dox: Doxorubicin

Table 3a: Urine analysis of mice in untreated control group administered with D/W (Group I)

Animal number	1	2	3	4	Normal range
Appearance	Yellow	Yellow	Yellow	Yellow	Pale to dark yellow
Urobilinogen	0.2	1.0	0.2	0.2	0.2-1.0
Ketone	-	±5	-	-	-
Blood	-	-	-	-	-
Protein	-	-	-	-	-
Nitrate	-	-	-	-	-
Leukocyte	-	-	-	-	-
Glucose	-	-	-	-	-
S.G.	1.025	1.025	1.03	1.025	1.005-1.030
pH	6.5	6	7	7	4.6-8.0

-: Negative; +: Positive

DISCUSSION

The current study reported the potential of HC9, a polyherbal formulation on tumor retardation and regulation of immunomodulatory cytokines in a mouse melanoma model. We have recently shown that HC9 demonstrated a significant anticancer activity against breast cancer cells through various mechanisms. Here, we have evaluated the effect of HC9 against melanoma. Various studies have reported an increased risk for skin cancer in patients having an earlier episode of breast cancer.[8] Female breast cancer survivors <45 years of age showed a 38% higher risk of developing melanoma as a second cancer than the general population.[9] Thus, newer drugs need to be explored for targeting not only breast cancer but also for preventing the recurrence of secondary cancers such as melanoma.

Interestingly, HC9 retarded tumor growth in mouse melanoma model. HC9 did not have any potential adverse effect on either mouse survival or organ pathology. Moreover, the tumor retardation potential of HC9 was comparable to that of the PC Dox. Our recent work has shown that HC9 modulated the expression of cell cycle, inflammation, and chromatin regulatory proteins. It decreased migration and invasion of breast cancer cells through modulation of matrix metalloproteinase (MMP)-2, MMP-9, hypoxia-inducible factor 1-alpha, and vascular endothelial growth factor expression.[7] We have reported earlier that HC9 contained picroside I, 6-gingerol, matairesinol, connesine, swertiamarin, berberine, and 2-hydroxy-4-methoxy-benzaldehyde found in *P. kurroa, C. rotundus, Z. officinale, C. deodara, T. cordifolia, H. antidysenterica, S. chirata, C. pareira,* and *H. indicus*, respectively.[5] Recently, these bioactives have been shown to decrease the viability of breast cancer cells (communicated). Moreover, compared to other bioactives, MA exhibited lower IC_{50} values. Thus, the anticancer activity of HC9 could be attributed to its component bioactives. Further, the individual plant materials of the formulation have been reported to exhibit anticancer activity. For example, *P. kurroa,*[10] *C. rotundus,*[12] *Z. officinale,*[13] *T. cordifolia,*[14] *H. indicus,*[15] and *C. deodara*[16] have been reported to retard the growth of Ehrlich ascites carcinoma-induced tumors in mice. *H. antidysenterica* has exhibited anticancer activity against human OVCAR-5 (ovary), HT-29 (colon), SK-N-MC (neuroblastoma), HEP-2 (liver), COLO-205 (colon), NIH-OVCAR-3 (ovary), and A-549 (lung) cell lines.[17] *S. chirata* has exhibited anticancer activity in DMBA-induced mouse skin carcinogenesis model.[18] *C. peltata* showed anticancer activity against human breast carcinoma cells.[19] All these studies suggested that the bioactives present in HC9 formulation could be regulating the melanoma growth through modulation of various signal transduction mechanisms.

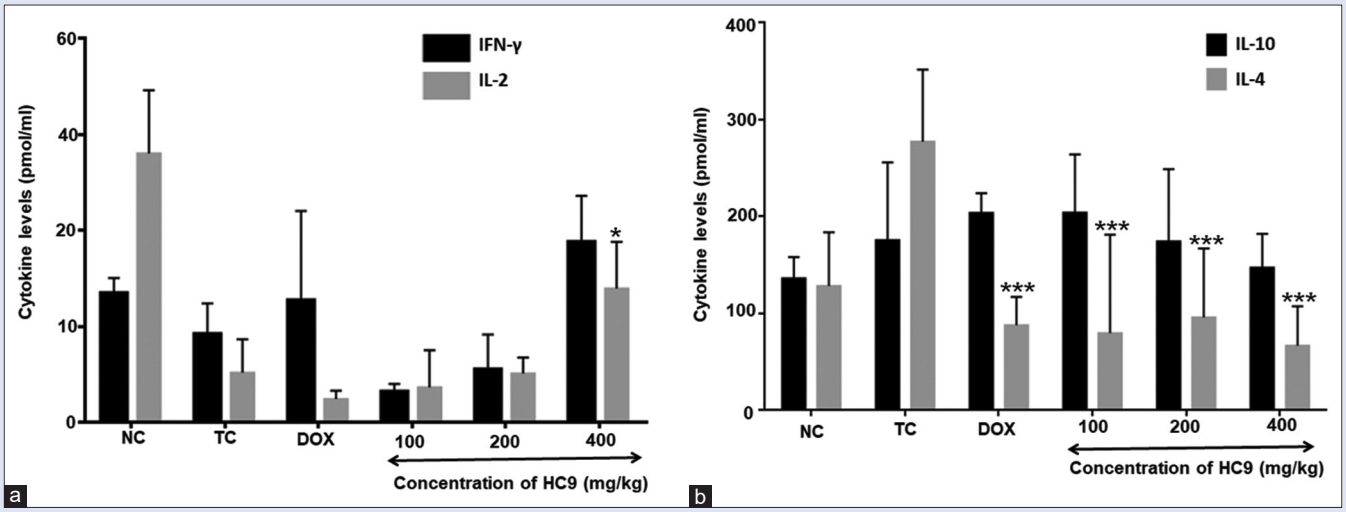

Figure 2: HC9-induced immunomodulation by regulating T-helper type 1/T-helper type 2 cytokines. Expression levels of interferon-γ (a) and interleukin-4 (b) cytokines in serum samples of mice from all the groups (tumor control, doxorubicin, and HC9). The data have been represented as mean ± standard deviation of mice from each group. The values with *$P < 0.05$ were considered to be statistically significant

Table 3b: Urine analysis of mice in untreated tumor control group administered with D/W (Group II)

Animal number	1	2	3	4	Normal range
Appearance	Yellow	Yellow	Yellow	Yellow	Pale to dark yellow
Urobilinogen	0.2	1.0	1.0	-	0.2-1.0
Ketone	-	-	-	-	-
Blood	-	-	-	-	-
Protein	-	-	-	-	-
Nitrate	-	-	-	-	-
Leukocyte	-	-	-	-	-
Glucose	-	-	-	-	-
S.G.	1.025	1.025	1.025	1.025	1.005-1.030
pH	6.5	6.5	7	7	4.6-8.0

-: Negative; +: Positive

Table 3c: Urine analysis of doxorubicin (2 mg/kg b.w.)-treated mice (Group III)

Animal number	1	2	3	4	Normal range
Appearance	Yellow	Yellow	Yellow	Yellow	Pale to dark yellow
Urobilinogen	1	0.2	1	0.2	0.2-1.0
Ketone	-	-	-	-	-
Blood	-	-	-	-	-
Protein	-	-	-	-	-
Nitrate	-	-	-	-	-
Leukocyte	-	-	-	-	-
Glucose	-	-	-	-	-
S.G.	1.03	1.025	1.025	1.025	1.005-1.030
pH	7	7	7	7	4.6-8.0

-: Negative; +: Positive

Various herbal formulations have been shown to exhibit anticancer activity against melanoma models. For example, a herbal composition having *Sophorae Flos* and *Lonicerae Japonicae Flos* has been reported to inhibit melanoma growth *in vivo*.[20] Other plants such as *Artemisia annua*,[21] *Hedyotis diffusa*,[22] and *Rosmarinus officinalis*[23] have also been reported to inhibit the proliferation, invasion, migration, and angiogenesis of melanoma cells *in vitro*. *Curcuma rhizome* exhibited antiproliferative and proapoptotic activities in B164A5 melanoma cells.[24] *Ganoderma lucidum* exhibited antimelanoma activity *in vitro* and *in vivo*, by inducing oxidative stress, apoptosis, and inhibition of cell proliferation.[25] *Coptidis rhizoma* water extracts or its major active chemical component, berberine, showed activity against human melanoma cells.[26] The coumarin fraction of *Cachrys pungens* Jan, was shown to be useful in the treatment of melanoma and nonmelanoma skin cancers.[27] Aerial components of *Ficus carica* L. cultivar Dottato (*F. carica*) exhibited antioxidant and phototoxic activities in human melanoma cells.[28]

HC9 modulated Th1 and Th2 cytokine response in the mice. These cytokines reflect immune response in various human diseases, including cancer.[29] Several studies have shown that there is a shift from Th1 to Th2 cytokines in cancer patients undergoing chemo- and radiotherapies that usually lead to immunosuppression.[30,31] Recently, plant extracts have been reported to modulate Th1 and Th2 expression in cancer.[32,33] HC9 upregulated IFN-γ and IL-2 (Th1) cytokines and reduced IL-10 and IL-4 (Th2) cytokine levels in mice. Resveratrol has been shown to improve the efficacy of IL-2 immunotherapy against melanoma model *in vivo*.[34] IFN-γ is an important lymphokine that can activate the immune cells (natural killer cells, cytotoxic T-lymphocytes, and tumoricidal macrophages), which preferentially attack breast cancer,[35] melanoma,[36] neuroblastoma,[37] and methylcholanthrene-induced tumors[38] by activation of CD8+ cytotoxic T-cells. IL-2 is a tumor suppressor/cytokine with pleiotropic effects on the immune system including monocytes and activated T-cells. IL-2 has been reported to suppress the growth of melanoma cells through activation of IL-24.[39] On the other hand, IL-4 and IL-10 contribute to tumor aggressiveness, induce immunosuppression, and help in avoiding tumor immune surveillance.[40,41] Some plants have been shown to inhibit the growth of melanoma cells through modulation of immune response. *T. cordifolia* has been shown to activate tumor-associated macrophages and induce antitumor activity in the spontaneous T-cell lymphoma.[42] *Astragalus membranaceus* has been shown to exhibit immune regulation by enhancing NK cell activity, inducing lymphocyte-mediated killing of tumors, and stimulating macrophage and B-cell activities.[43] Similarly, the aqueous extract of *Daphne gnidium* was reported to exhibit antitumor and immunomodulatory activities in mouse melanoma model.[44]

Table 3d: Urine analysis of mice treated with 100 mg/kg HC9 (Group IV)

Animal number	1	2	3	4	Normal range
Appearance	Yellow	Yellow	Yellow	Yellow	Pale to dark yellow
Urobilinogen	1	1	1	1	0.2-1.0
Ketone	-	-	-	-	-
Blood	-	-	-	-	-
Protein	-	Trace	-	-	-
Nitrate	-	-	-	-	-
Leukocyte	-	-	-	-	-
Glucose	-	-	-	-	-
S.G.	1.03	1.03	1.03	1.03	1.005-1.030
pH	7	7	7	7	4.6-8.0

-: Negative; +: Positive

Table 3e: Urine analysis of mice treated with 200 mg/kg HC9 (Group V)

Animal number	1	2	3	4	Normal range
Appearance	Yellow	Yellow	Yellow	Yellow	Pale to dark yellow
Urobilinogen	+	+	1	1	0.2-1.0
Ketone	-	-	-	-	-
Blood	-	-	-	-	-
Protein	-	-	-	-	-
Nitrate	-	-	-	-	-
Leukocyte	-	-	-	-	-
Glucose	-	-	-	-	-
S.G.	1.025	1.025	1.03	1.03	1.005-1.030
pH	7	7	7	7	4.6-8.0

-: Negative; +: Positive

Table 3f: Urine analysis of mice treated with 400 mg/kg HC9 (Group VI)

Animal number	1	2	3	4	Normal range
Appearance	Yellow	Yellow	Yellow	Yellow	Pale to dark yellow
Urobilinogen	1	1	1	0.2	0.2-1.0
Ketone	±5	±5	-	-	-
Blood	-	-	-	-	-
Protein	Trace	-	Trace	-	-
Nitrate	-	-	-	-	-
Leukocyte	-	-	-	-	-
Glucose	-	-	-	-	-
S.G.	1.03	1.03	1.03	1.025	1.005-1.030
pH	7	7	7	7	4.6-8.0

-: Negative; +: Positive

CONCLUSION

The current report indicated the antitumor and immunomodulatory potential of HC9 in melanoma model although it has been earlier reported to exhibit antibreast cancer activity. Herbal drugs that would target multiple cancers could be a boon to the patients as such drugs could prevent recurrence with second cancers.

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Conflicts of interest

There are no conflicts of interest.

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