



ORIGINAL ARTICLE

The flavonoid hesperidin methyl chalcone as a potential therapeutic agent for cancer therapy: Molecular docking, *in vitro* cytotoxicity, and *in vivo* antitumor activity



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Received 15 December 2022; accepted 27 February 2023

Available online 4 March 2023

KEYWORDS

Cancer;
Ehrlich ascites carcinoma;
Flavonoid;
Hesperidin methyl chalcone;
Molecular docking;
MTT assay

Abstract Hesperidin methyl chalcone (HMC) is a methylation product of the flavanone hesperidin, a flavonoid derived from citrus fruits. Many reports have emphasized the analgesic, anti-inflammatory and antioxidant properties of HMC. However, the anticancer potential of HMC has not been fully elucidated. The objective of this study was to assess the possible anticancer potential of HMC. MTT assay was carried out to assess the *in vitro* cytotoxicity of HMC against using A549 cancer cell line. In addition, the *in vivo* antitumor activity of HMC was screened against murine Ehrlich ascites carcinoma (EAC) model, in terms of tumor volume, tumor weight, life span,

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Peer review under responsibility of King Saud University.



hematological and biochemical parameters, and compared with that of the anticancer agent, hesperetin. HMC was efficient to suppress the cell viability of A549 cancer cells with an IC_{50} value of 51.12 μ M, which was comparable to that of the anticancer agent hesperetin ($IC_{50} = 49.12 \mu$ M). Similarly, in Ehrlich ascites carcinoma model, HMC significantly inhibited the growth of Ehrlich ascites carcinoma with mutual increase in the life span of HMC-treated mice, compared to EAC control. Most importantly, HMC showed a good safety profile as evidenced by restoring hematological profile count of RBCs, WBCs, and hemoglobin to the normal levels. HMC also efficiently reduced the oxidative stress in EAC-bearing mice via increasing the levels of GSH, SOD and Catalase. Collectively, HMC exerted a potent anticancer activity and data presented in this work suggest the usefulness of HMC as a promising candidate in cancer therapy.

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1. Introduction

Cancer is a fatal disease caused by uncontrolled cell proliferation and stands next to cardiovascular diseases in terms of mortality and morbidity (Nagai and Kim, 2017; Koene et al., 2016; Lila et al., 2014). Various strategies including surgery, chemotherapy, and radiation, employed alone or in combination, are currently adopted to mitigate cancer progression (Moin et al., 2021; Ando et al., 2015; Matlapudi et al., 2015; Abu Lila et al., 2009; Emam et al., 2018). However, multidrug resistance (MDR) and adverse effects are significant barriers to effective cancer therapy (Hussain et al., 2019). Accordingly, the search for safer and more effective chemoprevention and treatment strategies is mandatory for augmenting the overall therapeutic outcomes as well as improving patients' quality of life.

Recently, there has been a renewed interest for the use of phytochemicals in cancer therapy due to their safety, low toxicity, and readily availability (Hussain et al., 2018). Phytochemicals and their derivatives are viewed as intriguing solutions for enhancing therapy efficacy and alleviating adverse effects in cancer patients (Choudhari et al., 2019; Ranjan et al., 2019). Chalcones, members of the flavonoid family, are simple chemical scaffolds found in a variety of natural plant products such as vegetables, teas, and fruits (Karthikeyan et al., 2015; Singh et al., 2014; Zhou and Xing, 2015). They show structural heterogeneity and can act on a variety of pharmacological targets. Chalcone family members have attracted a lot of attention not only because of their synthetic and biosynthetic capabilities, but also because of their broad spectrum of biological activities, which include antioxidant (Lin et al., 2019), anti-inflammatory (Mahapatra et al., 2017), antimicrobial (Henry et al., 2020); antidiabetic (Rocha et al., 2020), cancer chemopreventive (Xu et al., 2015), and anticancer activities (Gao et al., 2020).

Hesperidin methyl chalcone (HMC) is a product of methylation of hesperidin, a flavonoid present mainly in citrus fruits (Nizamutdinova et al., 2008). Hesperidin has a low water solubility, leading in poor absorption in the small intestine; however, its solubility improves following an alkaline methylation process, which facilitates hesperidin isomerization and the formation of the HMC. Owing to its high-water solubility, HMC grants enhanced absorption, metabolic stability, and systemic bioavailability (Pinho-Ribeiro et al., 2015; Chanal et al., 1981). Many reports have emphasized the analgesic, anti-inflammatory and antioxidant properties of HMC (Pinho-Ribeiro et al., 2015; Guazelli et al., 2021). However, the anticancer potential of HMC has not been fully elucidated.

Since drug discovery and development are both time- and cost-consuming, they provide several hurdles for researchers working on drug design and discovery for diverse diseases such as various forms of cancer. As a result, the adoption of innovative technologies can open the way for the discovery of novel drugs with outstanding

therapeutic efficiencies, making a substantial contribution to disease treatment. Molecular docking is a cutting-edge computational drug design technology that predicts the most effective and stable state shape of the ligand-receptor complex (Morris and Lim-Wilby, 2008). Currently, molecular docking is considered one of the commonly adopted therapeutic approaches for anti-cancer drug designing.

In this present work, therefore, we aimed at investigating the possible anticancer potential of the flavonoid conjugated chalcone, hesperidin methyl chalcone (HMC). Molecular docking studies were conducted to target the catalytic site of the cancer specific enzymes, i.e., Polo-like kinase 1 (PLK1) and Epidermal Growth Factor Receptor tyrosine kinase domain (EGFR). Downregulation of both PLK1 and EGFR have been correlated-well with the tumor suppression, and thereby, designing of variety of robust PLK1 and EGFR inhibitors for cancer management is an upcoming strategy against cancer (Gunasekaran et al., 2022; Stratmann and Sebastian, 2019; Su et al., 2022; Alapati et al., 2012). Accordingly, in the present study, the in vitro anticancer activity of HMC was examined against A549 cancer cell line after completing molecular interaction analysis against PLK1 and EGFR. Furthermore, the in vivo antitumor efficacy of HMC was also examined in Ehrlich ascites carcinoma animal model.

2. Materials and methods

2.1. Materials

Hesperetin, hesperidin methyl chalcone and 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma Aldrich (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS) and antibiotic-antimycotic solution were procured from Gibco (Gaithersburg, MD, USA).

2.2. Cell lines

Human non-small cell lung cancer A549 cells and murine alveolar macrophages J774A.1 were obtained from National Centre for Cell Science (Pune, India). Ehrlich ascites carcinoma (EAC) cells were provided by the Amala Cancer Research Centre (Thrissur, Kerala). A549 cells were cultured in RPMI-1640 medium while J774A.1 were cultured in Dulbecco's modified Eagle medium (DMEM). Both media were supplemented with heat-inactivated 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin incubated in a humid atmosphere (5% CO_2 /95% humidified air).

2.3. Experimental animals

Swiss albino mice (20–25 g) were obtained from Central Animal Facilities, Acharya & BM Reddy College of Pharmacy, Bengaluru, India. Animals were housed under standard temperature- and humidity conditions, and they had free access to mouse chow and water. The Institutional Animal Ethics Committee at Acharya & BM Reddy College of Pharmacy authorized the study protocol (IAEC/ABMRCP/2017–2018/17).

2.4. In silico molecular docking

2.4.1. Target protein and ligand preparation

Protein data bank was used to obtain the 3D structures of target proteins PLK1 (PDB ID: 2OWB) and EGFR (PDB ID: 1 M17) in pdb format. However, each protein was analyzed by Discovery Studio Visualizer tool and heteroatoms were removed. Further, both the target proteins were converted from.pdb to.pdbqt format with the help of AutoDock 4.2 tool by applying the protocol of Rizvi et al. (Rizvi et al., 2013). Kollman united atom charges, solvation parameters and polar hydrogen were also added to the target protein via AutoDock 4.2 before converting them into.pdbqt format.

Three-dimensional structures of PHA-680626 (native ligand for PLK1), Erlotinib (native ligand for EGFR) and Hesperetin (control) were retrieved from the ChemSpider database by using ID 10137725, 154,044 and 65234, respectively. On the other hand, HMC (ID 6436550) 3D structure was obtained from PubChem database. Moreover, OpenBabel tool was used to convert all the ligand structures into.pdbqt format prior to docking.

2.4.2. Docking experiment

AutoDock Vina tool [<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3041641/>] was used to dock each ligand to the target proteins by using a protocol described by Moin et al. (Moin et al., 2022). Pre-defined grid coordinates were used to target both the proteins active sites, i.e., x: 0.069; y: 23.58; z: 66.741 for PLK1 enzyme and x: 24.379; y: 0.093; z: 56.141 for EGFR, respectively. However, grid box size was kept as 40 × 40 × 40 Å for both target proteins. Affinity in kcal/mol was obtained for each ligand-target protein interaction, and the algorithm presented results in descending order (10 modes). Further, complex of best docking confirmation was obtained by using PyMol tool, and detailed interaction analysis was done on discovery studio visualizer. Moreover, LigPlot analysis was also performed for each complex to get deeper insight of the interaction. It is noteworthy to mention that both the target proteins were re-docked with native ligand to validate the protocol before performing the docking experiments.

2.5. In vitro cytotoxicity assay

The MTT assay was adopted to estimate the number of viable cells. Briefly, 1×10^4 A549 cells were seeded per well in 96-well microtiter plates. The cells were treated with different concentrations (6.25, 12.5, 25, 50, and 100 μM) of either HMC or the anticancer agent, hesperetin and the plates were incubated at

37 °C. After 24 h of incubation, 10 μL of MTT solution (5 mg/mL) was added to each well and the plates were further incubated at 37 °C for 4 h. The formed formazan crystals were then dissolved in 100 μL of acid-isopropanol (0.04 N HCl in isopropanol). The absorbance was measured at a wavelength of 570 nm using an ELISA plate reader.

2.6. In vivo studies

2.6.1. Development of Erlich ascites carcinoma model

Erlich ascitic carcinoma (EAC) cells (1×10^6 cells) were transplanted into the peritoneal cavity of a Swiss albino mouse to propagate. After 16 days, the donor mouse received an intraperitoneal injection of 0.1 mL of normal saline (0.9% w/v) and 1 mL of ascites fluid was collected immediately from the peritoneal cavity and diluted to 10 mL with normal saline. 10 μL of ascites fluid were then placed in Neubauer's chamber and counted by a hemocytometer.

2.6.2. Experimental design

On day zero, 1×10^6 EAC cells were intraperitoneally injected in Swiss albino mice. 24 h later, the inoculated mice with Ehrlich cells were randomly divided into three groups (n = 12). The first group received phosphate buffer saline and served as EAC-bearing control group. The second group was treated with hesperetin (30 mg/kg) (Aranganathan et al., 2009). The third group was treated with HMC (30 mg/kg). HMC dose was chosen based on previous work (Guazelli et al., 2021). Treatments were given i.p. at 24 h post tumor inoculation and continued once daily for 15 days. On day 16, six mice from each group were euthanized for the estimation of tumor growth, hematological, and hepatic anti-oxidative parameters. The remaining mice in each group were kept alive and monitored for survival parameters.

2.6.2.1. Estimation of tumor volume. The ascitic fluid was collected from peritoneal cavity of each mouse and the tumor volume was estimated by comparing the volume of ascetic fluid

Table 1 Molecular docking results of different ligands with PLK1.

Ligands	Free Binding Energy	Interacting Amino Acids
PHA-680626 (Redocked Native Ligand)	−9.0 kcal/mol	Arg57, Leu59 , Ala80, Val114, Glu131, Leu132, Cys133, Arg134 , Arg136, Phe183, Glu186
HMC	−8.5 kcal/mol	Leu59, Gly60, Lys61, Gly62, Ala65, Lys66, Cys67, Ala80, Lys82, Val114, Glu131 , Leu132, Cys133 , Arg136 , Ser137, Leu139, Glu140 , Lys178 , Gly180, Phe183
Hesperetin	−7.8 kcal/mol	Leu59, Cys67, Ala80, Val114, Leu130, Glu131 , Leu132, Cys133 , Arg136, Ser137, Leu139, Glu140 , Gly180, Phe183

*Hydrogen bonded amino acids are presented in bold.

present in the peritoneal cavity of treated groups to those in the control group as on day 16 of the experiment.

2.6.2.2. Hematological parameters. To assess the impact of HMC treatment on hematological parameters in EAC-bearing mice, blood samples were collected from *retro*-orbital venous plexus into EDTA Eppendorf tubes. Hematological parameters such as hemoglobin (Hb) content, red blood cells (RBCs) and white blood cells (WBCs) counts were estimated.

2.6.2.3. Hepatic antioxidant parameters. The liver of each mouse was dissected, washed with cold saline, and blotted on filter paper. A definite weight of 1 g of liver tissue was then homogenized with 10 mL cold PBS (0.2 M, pH 7) at 4000 rpm. Liver homogenates were then centrifuged at 10,000 rpm at 4 °C for 15 min. The collected supernatant was subjected to different biochemical estimations such as Superoxide dismutase (SOD), glutathione synthetase (GSH), catalase (CAT), Nitric oxide (NO) (Sinha, 1972; Habig et al., 1974; Kakkar et al., 1984).

2.6.2.4. Survival parameters. The effect of Hesperetin (30 mg/kg) and HMC (30 mg/kg) on tumor growth was monitored in terms of median survival time (MST) and percentage increase in life span (% ILS). MST was estimated by recording the mortality rate daily until all the animals were dead and % ILS was calculated using the following formula (Saad et al., 2017):

$$\%ILS = \left[\frac{MST \text{ of treated group}}{MST \text{ of control group}} - 1 \right] \times 100$$

2.6.2.5. Weight variation parameter. To estimate the differences in body weight between the control EAC group and treated groups, animals were weighed every other day from day zero.

2.7. Statistical analysis

All the data are expressed as mean \pm SD. GraphPad Prism 7 software was employed to analyze the data using one-way ANOVA followed by Dunnett's *t*-test for multiple comparisons. **P* < 0.05 was considered significant.

3. Results and discussion

3.1. *In silico* studies

Molecular docking interaction experiments were performed to target the native ligand, HMC and Hesperetin on both the proteins (PLK1 and EGFR) by using AutoDock Vina. Table 1 and Fig. 1 shows the docking interaction results of native ligand (PHA-680626, a selective PLK inhibitor), HMC and Hesperetin (control) on the catalytic site of PLK1. To validate the protocol re-docking experiment with native ligand was performed on PLK1; interestingly, the re-docked native ligand bound to the same active site cavity as the native ligand in the crystal structure (Kothe et al., 2007). The superimposition image (Fig. 1a) confirms the standardization of the protocol. In fact, HMC and hesperetin also bounded to the same catalytic site as native ligand (Fig. 1b). Detailed interaction anal-

ysis showed that native ligand once re-docked with PLK1 active site showed binding energy of -9.0 kcal/mol, and eleven amino acids of PLK1 (Arg57, Leu59, Ala80, Val114, Glu131, Leu132, Cys133, Arg134, Arg136, Phe183, Glu186) were involved in this interaction (Table 1; Fig. 1c and d). Among these amino acids, Leu59 and Arg134 showed strong hydrogen bonding interaction with the native ligand. Whereas HMC showed -8.5 kcal/mol binding energy with the involvement of twenty amino acids of PLK1 'active site' in the interaction (Table 1; Fig. 1e and f). Interestingly, HMC showed strong hydrogen bonding with five amino acids (Glu131, Cys133, Arg136, Glu140, Lys178) of PLK1 catalytic site during 'HMC-PLK1' molecular interaction. On the other hand, 'hesperetin-PLK1' interaction showed binding energy of -7.8 kcal/mol, and three hydrogen bonding with Glu131, Cys133 and Glu140 amino acid residues of PLK1 (Table 1; Fig. 1g and h). It has been reported by Shakil et al (Shakil et al., 2019) that six-amino acids, namely, Leu59, Cys67, Leu130, Cys133, Arg136 and Phe183 are crucial for binding of inhibitors to the kinase domain of PLK1. They also revealed that Cys67 and Phe183 forms the top and bottom of the ATP-binding pocket of PLK1, respectively; however, Leu132 forms its hinge-region. In the present study, among the important kinase domain amino acids of PLK1, HMC interacted with Cys133 and Arg136, hesperetin interacted with Cys133 and native ligand interacted with Leu59 through strong hydrogen bonds. Moreover, HMC has shown interaction with all the crucial amino acids of PLK1 mentioned in the Shakil et al (Shakil et al., 2019) report via hydrogen or hydrophobic interactions. These results confirmed the strong bonding of our targeted molecule (HMC) against a potent anticancer target PLK1. section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

Similar approach was applied on another anti-cancer target i.e., EGFR. Table 2 and Fig. 2 shows interaction of native ligand (erlotinib), HMC and hesperetin with EGFR. Superimposition of native ligand and re-docked native ligand validated the protocol (Fig. 2a). However, superimposition of all the docked structures of EGFR confirmed that HMC and hesperetin have also bounded to the same site as native ligand (Fig. 2b). Native ligand showed binding energy of -7.4 kcal/mol and two hydrogen bonding (Met769 and Cys773) with EGFR (Table 2; Fig. 2c and d). Whereas HMC showed better binding energy of -9.3 kcal/mol with five hydrogen bonding (Lys721, Glu738, Met742, Met769 and Arg817) with EGFR (Table 2; Fig. 2e and f). Similarly, hesperetin also showed five hydrogen bonding (Lys721, Glu738, Leu764, Thr830 and Asp831) with EGFR; however, the binding energy was -8.5 kcal/mol (Table 2; Fig. 2g and h). The result indicated strong interaction of HMC with EGFR better than the native ligand and control hesperetin. In a 2017 study (Puranik and Srivastava, 2017), rugosafavonoid showed strong anticancer activity against breast cancer, and Met769 amino acid of EGFR was common in binding with all the derivatives of rugosafavonoid. In a subsequent investigation, Asn818, Lys721, Leu694, Val702 and Met742 were considered as crucial active site residues for EGFR binding with inhibitor (Yan et al., 2018). Recently, the importance of Glu738, Met742, Asp831 and Arg817 in binding with inhibitors has also been explored (Al-Warhi et al., 2022). Interestingly,

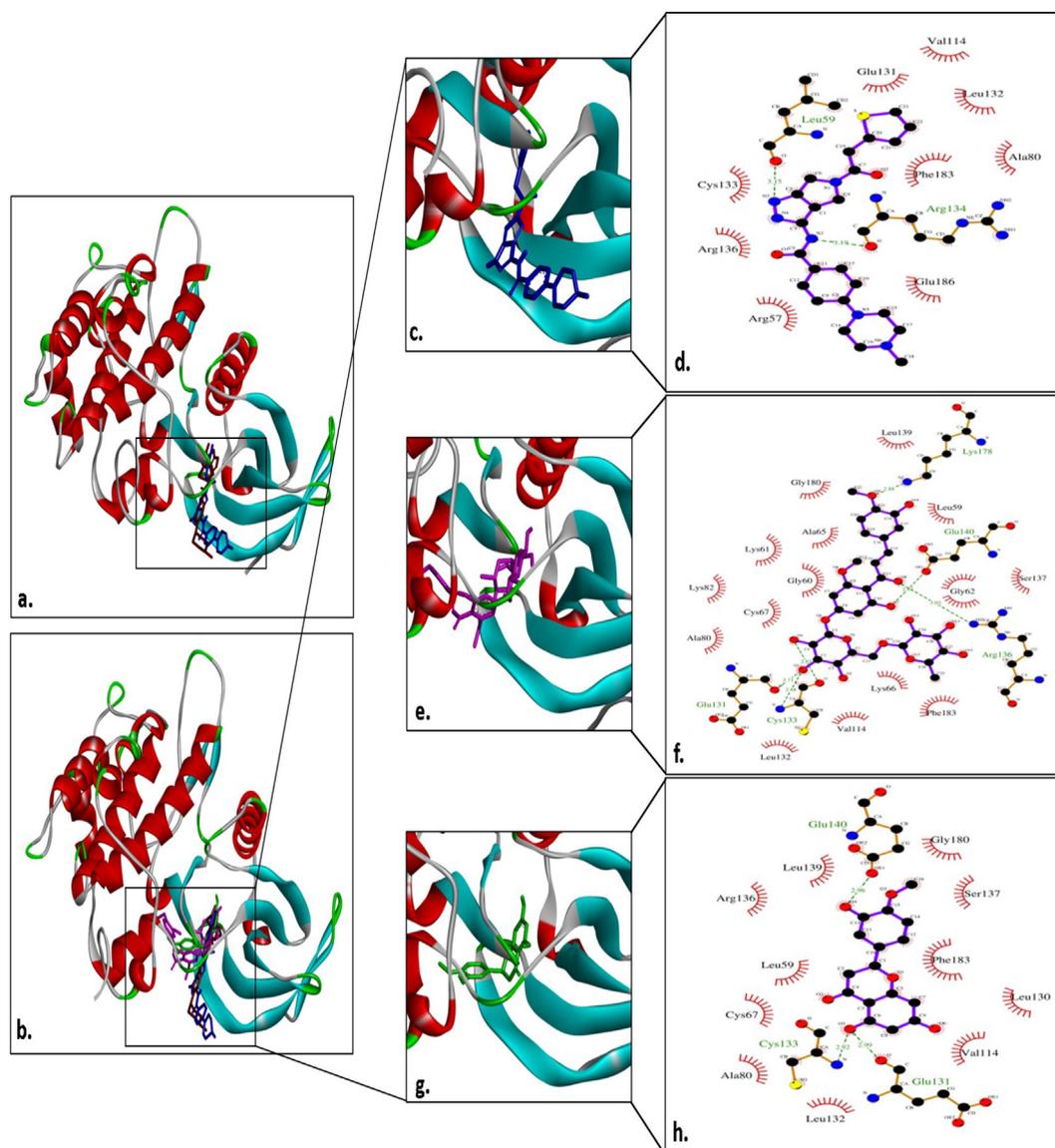


Fig. 1 Interaction analysis of different ligands with PLK1. (a) Superimposition image of native ligand (brown) and re-docked native ligand (blue) after binding to PLK1 active site; (b) Superimposition image of all the ligands [native ligand (brown); re-docked native ligand (blue); HMC (magenta) and hesperitin (green)] after binding to PLK1 active site; (c-d) Interaction of re-docked native ligand with PLK1 and its LigPlot analysis; (e-f) Interaction of HMC with PLK1 and its LigPlot analysis; and (g-h) Interaction of hesperitin with PLK1 and its LigPlot analysis.

HMC showed strong hydrogen bonding with Lys721, Glu738, Met742, Met769 and Arg817 that have been reported as crucial active site amino acids for EGFR.

Overall, in-silico results indicated that our target molecule 'HMC' could interact strongly with PLK1 as well as EGFR, and has a potential to develop into an anticancer molecule. It has been observed that in-silico findings, correlate-well with the wet lab investigations and provide a preliminary idea about the tested molecule. However, in vitro and in vivo validations are necessary to conclude any in-silico findings. Hence, HMC has been further tested against A549 cells and Ehrlich ascites carcinoma. In fact, there are several reports pertinent to the development of PLK1 and EGFR inhibitors against both these cancer types (Gunasekaran et al., 2022; Stratmann and

Sebastian, 2019; Su et al., 2022). Thus, the further in vitro and in vivo investigation has its due clinical relevance.

3.2. In vitro cytotoxicity of HMC against A549 cells

To assess the inhibitory effects of HMC on the cell viability of A549 cells, the cells were exposed to serial dilutions of HMC (0, 6.25, 12.5, 25, 50 and 100 μ M) for 24 h and the cell viability were evaluated by MTT assay and compared with that of hesperetin. As shown in Fig. 3A, HMC remarkably inhibited the growth of A549 in a dose-dependent manner. The cell viability of A549 cells was declined from $80.53 \pm 3.17\%$ to $12.78 \pm 1.82\%$ upon increasing HMC concentration from 6.25 to 100 μ M. The calculated IC_{50} value of HMC against A549 cells

Table 2 Molecular docking results of different ligands with EGFR.

Ligands	Free Binding Energy	Interacting Amino Acids
Erlotinib (Re-docked native ligand)	-7.4 kcal/mol	Leu694, Val702, Ala719, Lys721, Leu764, Thr766, Gln767, Met769 , Pro770, Phe771, Gly772, Cys773 , Leu820, Thr830, Asp831
HMC	-9.3 kcal/mol	Leu694, Phe699, Val702, Ala719, Lys721 , Glu738 , Met742 , Leu764, Thr766, Leu768, Met769 , Gly772, Arg817 , Leu820, Thr830, Asp831, Gly833
Hesperetin	-8.5 kcal/mol	Gly695, Phe699, Val702, Ala719, Ile720, Lys721 , Glu738 , Met742, Leu764 , Thr766, Leu820, Thr830 , Asp831

*Hydrogen bonded amino acids are presented in bold.

was $51.16 \pm 1.9 \mu\text{M}$, which was comparable to that of a the cytotoxic agent, hesperetin ($\text{IC}_{50} 49.14 \pm 1.4 \mu\text{M}$). These results suggest the potent cytotoxic potential of HMC against non-small cell lung cancer A549 cells.

Finding cytotoxic drugs with great selectivity for cancer cells is critical for improving the survival rates of cancer patients. Consequently, the cytotoxicity of HMC was screened against noncancerous murine alveolar macrophages J774A.1 cells, and the selectivity index was calculated. As shown in Fig. 3B, insignificant decrease in the viability of J774A.1 cells was observed upon treatment with lower HMC concentration (up to $200 \mu\text{M}$). Nevertheless, at relatively higher concentrations (400 and $800 \mu\text{M}$), HMC could elicit a pronounced cytotoxic effect against J774A.1 cells. The IC_{50} value of HMC against J774A.1 cells was $649.7 \pm 23.4 \mu\text{M}$. Most importantly, the calculated selectivity index (S.I.), obtained by dividing the average of the IC_{50} value in the noncancerous cell line (J774A.1) by the IC_{50} value in the cancer cell line (A549), was found 12.07, indicating the great selectivity of HMC towards cancerous cell line rather than normal noncancerous cells.

3.3. In vivo antitumor activity of HMC in Erlich ascites carcinoma (EAC) model

Ehrlich ascites carcinoma (EAC), a murine mammary adenocarcinoma, is a rapidly developing undifferentiated malignancy with aggressive behavior (Jaganathan et al., 2010), capable of growing in almost all mice strains (Mishra et al., 2018), and is widely employed in cancer research. In this study, in order to address the anti-tumor potential of HMC against EAC cells, changes in ascitic fluid volume of treated mice were assessed at the end of day 16. Generally, ascitic fluid is a crucial nutritional requirement for the growth of EAC cells, consequently, a rapid rise in ascitic fluid is considered a key indicator for tumor progression (Haldar et al., 2010). As depicted in Fig. 4A, treatment of EAC-bearing mice with HMC for 15 consecutive days significantly decreased ascitic fluid volume, compared to EAC untreated control mice. Furthermore, the antitumor effect of HMC, in terms of ascitic fluid volume, was similar to that of hesperetin.

Body weight of EAC-bearing mice is another important parameter for estimating the anticancer potential of MHC. Animal body weights were measured on a regular basis on predetermined days. As shown in Figure 4B, a remarkable rise in body weight of the animals was detected in positive control mice, presumably, due to the rapid and progressive accumula-

tion of ascites tumor cells. On the other hand, a significant drop in in body weights was observed in animals treated with either HMC or hesperetin, compared to EAC control mice. The percent decrease in body weight observed upon treatment with either HMC or hesperetin were 20.37% and 22.59%, respectively.

A mounting body of evidences have revealed that prolongation of life span of tumor bearing animals, along with a decrease in tumor volume are considered reliable criteria for depicting the potential of any anticancer agent (Haldar et al., 2010; Dolai et al., 2012; Ghosh et al., 2011). Herein, treatment with HMC for 15 days significantly prolonged the survival of EAC-bearing mice, compared to EAC-bearing untreated mice (Fig. 4C). The median survival time of mice treated with either HMC or hesperetin significantly increased to 30.5 ± 1.1 days (ILS = 73.78%), and 32 ± 1.9 days (ILS = 78.41%), respectively, when compared to EAC control group (MST 17.5 ± 0.5 days). Collectively, HMC efficiently exerted a potent antitumor activity in EAC-bearing mice as evidenced by a significant decrease in tumor volume, tumor weight, along with a remarkable increase in life span, compared to EAC-bearing control mice.

3.4. Effects of HMC on hematological parameters

The most common complications associated with cancer treatment are myeloid suppression and anemia caused by a decrease in red blood cells (RBCs) or hemoglobin (Hb) content (Opore Kennedy et al., 2001). As summarized in Table 3, EAC-bearing mice had lower RBCs count (2.16 ± 0.34 million/ mm^3) and Hb content (4.66 ± 0.73 g/dL), which might be attributed, on the one hand, to the suppressive effect of EAC on bone marrow erythropoiesis (DeGowin and Gibson, 1978), and on the other hand, to iron deficiency in a myelopathic or hemolytic condition (Saad et al., 2017). On interest, EAC-bearing animals treated with HMC or the anticancer agent, hesperetin, showed significant increases ($p < 0.001$) in RBCs count and Hb content toward normal. RBCs counts in HMC- and hesperetin-treated mice were 8.56 ± 0.59 million/ mm^3 and 7.43 ± 0.62 million/ mm^3 , respectively, and Hb contents in HMC- and hesperetin-treated mice were 10.66 ± 0.54 g/dL and 11.7 ± 0.57 g/dL, respectively. These enhancements clearly show that HMC has a protective effect on the haemopoietic system.

One of the most significant criteria for assessing the therapeutic effectiveness of anticancer medications is their ability to lower white blood cells (WBCs) count in tumor-bearing mice.

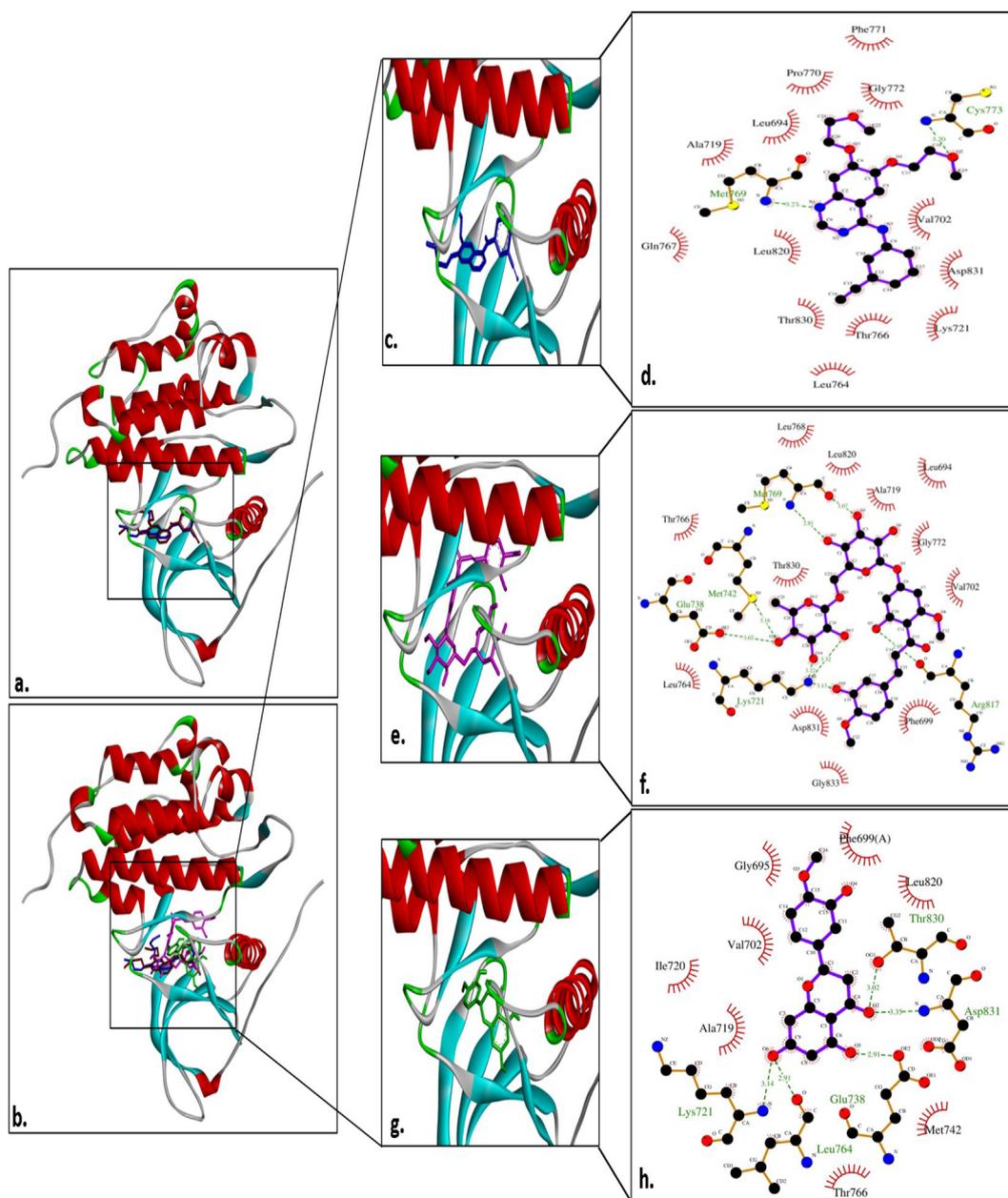


Fig. 2 Interaction analysis of different ligands with EGFR. (a) Superimposition image of native ligand (brown) and re-docked native ligand (blue) after binding to EGFR active site; (b) Superimposition image of all the ligands [native ligand (brown); re-docked native ligand (blue); HMC (magenta) and hesperetin (green)] after binding to EGFR active site; (c-d) Interaction of re-docked native ligand with EGFR and its LigPlot analysis; (e-f) Interaction of HMC with EGFR and its LigPlot analysis; and (g-h) Interaction of hesperetin with EGFR and its LigPlot analysis.

Generally, chemotherapy not only destroys rapidly divided cancer cells but could also kills some rapidly dividing normal cells in the body, such as those in bone marrow that make WBCs. Accordingly, a transient drop in WBC levels is generally observed with chemotherapy treatments. Herein, as depicted in [Table 3](#), the level of WBCs was increased in EAC control mice (53330 ± 1770 cells/mm³). On the other hand, treatment with either HMC or hesperetin effectively ($p < 0.001$) restored WBCs count to the normal level, when compared to EAC control group. Collectively, these results evidently emphasized HMC had hematopoietic protective

function without causing myelotoxicity, the most prevalent adverse effect of cancer treatment.

3.5. Effects of HMC on antioxidant parameters

Glutathione (GSH) and antioxidant enzymes, catalase (CAT) and superoxide dismutase (SOD), play vital roles in the antioxidant defense mechanisms, via mitigating the harmful effects of free radicals, during carcinogenesis ([Rajkapoor et al., 2007](#); [Singh and Ahluwalia, 2002](#)). Imbalance in endogenous antioxidant enzymes along with increased free radical production are

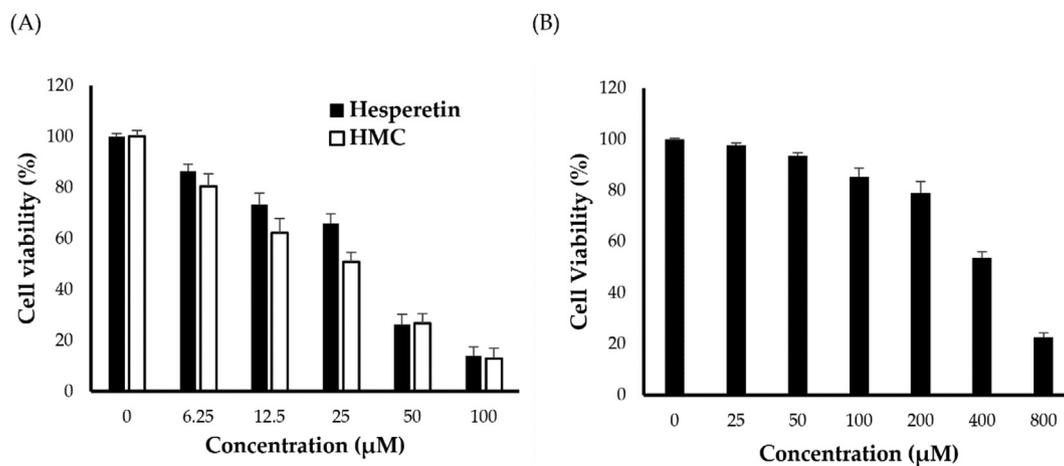


Fig. 3 (A) *In vitro* cytotoxicity of HMC and hesperetin against A549 cancer cells; (B) *In vitro* cytotoxicity of HMC against noncancerous murine alveolar macrophages J774A.1 cells.

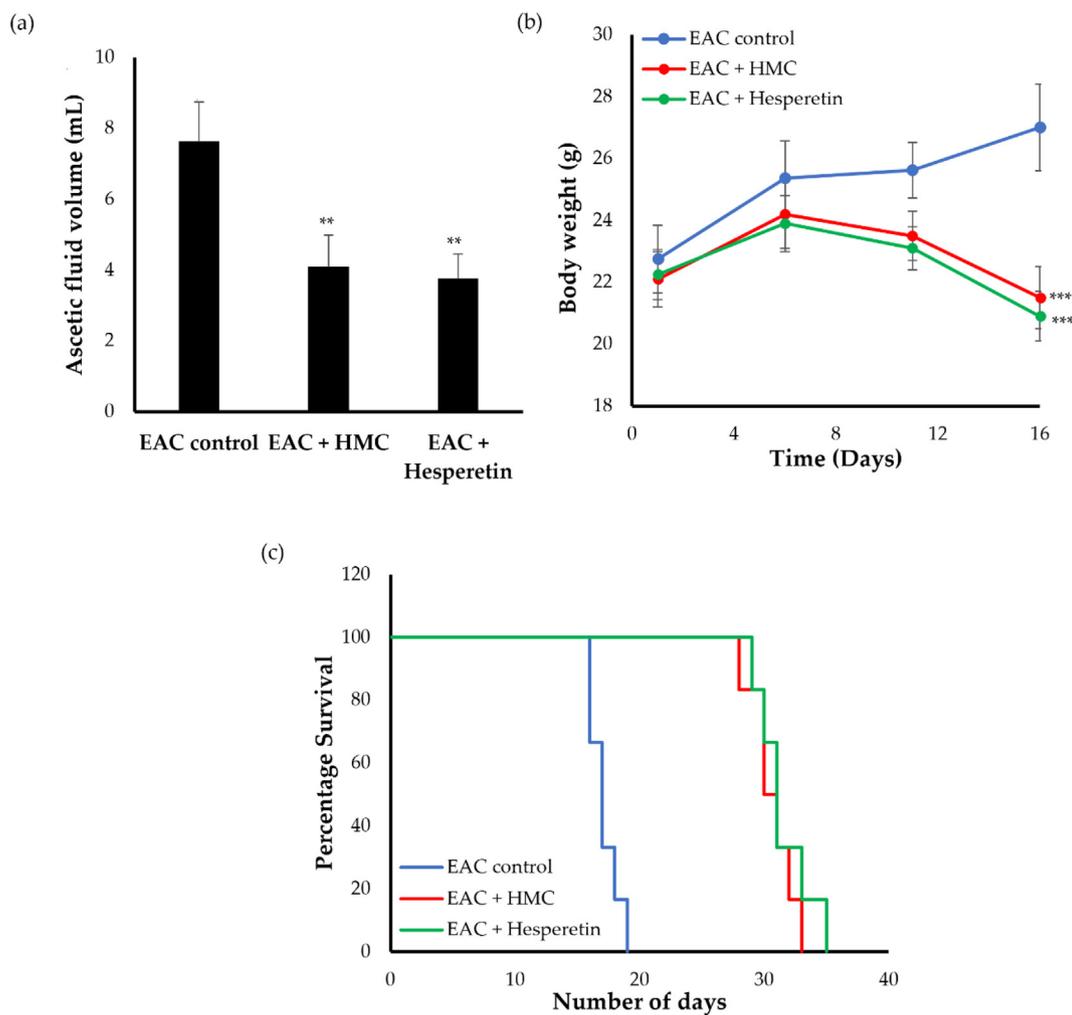


Fig. 4 Effect of HMC and hesperetin on (a) ascetic fluid volumes; (b) body weight and (c) median survival time in EAC-bearing mice. Data were expressed as mean \pm SD (n = 6). ** P < 0.01, *** P < 0.001 vs. EAC control mice.

Table 3 Effects of HMC on Hb, RBC and WBC (Hematological parameters).

Groups	Hb (g/dL)	RBC ($1 \times 10^6 / \text{mm}^3$)	WBC ($1 \times 10^3 / \text{mm}^3$)
Normal control	10.98 \pm 0.89	7.61 \pm 0.95	16.7 \pm 0.61
Cancer Control	4.66 \pm 0.73	2.16 \pm 0.34	53.33 \pm 1.77
HMC	10.66 \pm 0.54***	8.56 \pm 0.59***	15.03 \pm 1.89***
Hesperetin	11.7 \pm 0.57***	87.43 \pm 0.62***	14.37 \pm 1.31***

Data represent mean \pm SD (n = 6). ****p* < 0.001 vs EAC-bearing control mice.

Table 4 Effects of HMC on antioxidant parameters.

Groups	SOD (IU/mg protein)	GSH (mM/g)	Catalase ($\mu\text{M}/\text{mg}$ protein)	Nitric oxide (mM/mL)
Normal Control	1.85 \pm 0.16	802.15 \pm 24.1	0.043 \pm 0.010	50.01 \pm 3.8
Cancer Control	1.04 \pm 0.19	153.6 \pm 18.9	0.012 \pm 0.005	21.19 \pm 1.6
HMC	1.95 \pm 0.11**	754.75 \pm 32.3**	0.028 \pm 0.007**	43.00 \pm 4.4**
Hesperetin	1.99 \pm 0.15**	869.57 \pm 48.8**	0.035 \pm 0.009**	46.23 \pm 2.5**

All the values were expressed as mean \pm SD (n = 6). ***P* < 0.01 vs. EAC-bearing control mice.

considered hallmarks in carcinogenesis (Szatrowski and Nathan, 1991). In this study, a significant decrease in hepatic antioxidants enzymes levels was detected in EAC-bearing control mice, suggesting the existence of an oxidative stress state, which might predispose hepatocytes injury (Table 4). Interestingly, treatment with either HMC or hesperetin significantly elevated GSH content ($p < 0.01$), CAT levels ($p < 0.01$), and SOD levels ($p < 0.01$) in liver tissues of the treated animals compared to EAC control mice (Table 4). The elevated levels of these enzymes can protect liver tissues from oxidative stress by scavenging superoxide radicals and the resulting H_2O_2 ; which constitute the substrates of SOD and CAT enzymes, respectively. Accordingly, these results emphasized the potential of HMC to reduce oxidative stress in EAC-bearing mice by regulating antioxidant levels. Similar findings were reported by Samudrala et al. who attributed the antitumor efficacy of *Alternanthera brasiliana* extract against Ehrlich ascites carcinoma to the potent antioxidant and hemoprotective nature of the extract.

Nitric oxide (NO) plays a crucial role in both the progression and suppression of carcinogenesis depending on its concentration (Khan et al., 2020). Generally, a high concentration of NO may trigger cancer cell apoptosis, and thereby, inhibit tumor growth. On the other hand, at low concentration of NO, cancer growth and proliferation are promoted (Xu et al., 2002). In this study, NO levels were significantly depressed (21.19 ± 1.6 mM/mL) in EAC-bearing control group, which might support cancer growth and proliferation. On the other hand, treatment with HMC efficiently restored the level of NO to normal levels, as compared to the EAC-bearing control group ($P < 0.01$). The NO levels in both HMC- and hesperetin-treated mice were 43.00 ± 4.4 and 46.23 ± 2.5 mM/mL, respectively. Collectively, the antitumor efficacy of HMC might be attributed, at least in part, to the efficient antioxidant potential observed in EAC model.

4. Conclusions

In this study the anticancer activity of hesperidin methylated chalcone (HMC) was evaluated in silico against PLK1 and EGFR cancer targets, in vitro against A549 cancer cells and in vivo in Ehrlich ascites

carcinoma (EAC) animal model. HMC showed strong interaction with the active site of both the cancer targets. HMC exhibited remarkable in vitro cytotoxicity against A549 cancer cell line. In addition, HMC therapy reduced tumor volume, tumor weight, and increased the life span of EAC-bearing mice. The potent antitumor activity detected in this model was correlated to the antioxidant potential of HMC, and was comparable to that of the reference agent, hesperetin. Furthermore, HMC showed a good safety profile as evidenced by restoring hematological profile count of RBCs, WBCs, and hemoglobin to the normal levels. Collectively, HMC might represent a novel candidate in cancer therapy with high antitumor efficiency and reasonable safety profile.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This research has been funded by Scientific Research Deanship at University of Ha'il - Saudi Arabia through project number RG-21 061.

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