HHS Public Access

Author manuscript

Colloids Surf B Biointerfaces. Author manuscript; available in PMC 2017 December 28.

Published in final edited form as:

Colloids Surf B Biointerfaces. 2017 May 01; 153: 208–219. doi:10.1016/j.colsurfb.2017.01.038.

Honokiol nanomicellar formulation produced increased oral bioavailability and anticancer effects in triple negative breast cancer (TNBC)

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Abstract

Triple negative breast cancer (TNBC), owing to its aggressive behavior and toxicity associated with available chemotherapy; currently no suitable therapy is available. Honokiol (HNK) is a promising anticancer drug but has poor bioavailability. In the current study, we evaluated the anticancer effects of an oral Honokiol nanomicellar (NM) formulation (size range of 20–40 nm) *in vitro* against various TNBC cells lines. Cytotoxicity, clonogenic and wound healing assays demonstrated the promising anticancer effects. *In vitro* Caco-2 permeability studies suggested increased absorption of Honokiol. Compared to HNK-FD, nanomicellar formulations resulted in significant increase in the oral bioavailability. C_{max} (4.06 and 3.60-fold) and AUC (6.26 and 5.83-fold) were significantly increased in comparison to oral 40 and 80 mg/kg free drug respectively. Further, anticancer effects of these formulations were studied in BALB/c nude mice transplanted with orthotopic MDA-MB-231 cell induced xenografts. After 4 weeks of daily administration of HNK-NM formulation, significant reduction in the tumor volumes and weights compared to free drug ($p < 0.001$) treated groups was observed. Surprisingly, in some of the animals (25%), the treatment resulted in complete eradication of tumors. Increased apoptosis and antiangiogenic effect was observed in HNK-NM groups compared to free drug and untreated control animals. This is the first report demonstrating that HNK-FD possesses anticancer effects against TNBC.

Keywords

Anticancer; Bioavailability; Honokiol; Nanomicellar formulation; TNBC

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Author contributions

CG and RD conceived the idea, performed all the experiments, collected, wrote manuscript and analyzed the data. AK participated in manuscript writing. MS conceived the idea, wrote the manuscript and approved the final version of manuscript.

1. Introduction

Triple negative breast cancer (TNBC) is an aggressive phenotype of breast cancer and due to lack of suitable targets; this form of breast cancer is difficult to treat [1–3]. However, recent research in many laboratories is focused on the use of natural products to combat cancer [4,5]. The reason to head towards the natural products arena is that most of the natural product based anticancer compounds are safer when compared to existing standard anticancer agents. Further, these agents are found to be available in various dietary supplements; therefore, these natural products at early stages of cancer may act as chemopreventive agents [6,7]. Though natural products have been reported for promising anticancer activities, most of these studies were based on parenteral administration. On the other hand, oral therapy with natural product based anticancer agents is found to be very difficult due to their limited bioavailability. Recent studies demonstrated that Honokiol (HNK) (a lignan compound isolated from bark of *Magnolia* plant) has multiple pharmacological effects [8,9]. Honokiol is a phytochemical agent isolated from the *Magnolia* plants (*Magnolia officinalis* and *Magnolia grandiflora*), and has antioxidant, anti-inflammatory and anticancer activities [10–12]. Chemically HNK is 2-(4-hydroxy-3-prop-2-enyl-phenyl)-4-prop-2-enyl-phenol and has been used in traditional Chinese and Japanese medicine system. Promising anticancer effects of HNK have been demonstrated in several cancer types [13–19]. It has also been reported to have anti-angiogenic and anti-oncogenic properties against diverse types of tumors [20,21]. HNK showed beneficial effects in different pathological conditions including neuropharmacological effects. Most of these diverse pharmacological effects are reported with intraperitoneal route of administration. However, due to poor aqueous solubility, HNK possesses limited oral bioavailability limiting its use in various diseases. Owing to poor oral bioavailability, the dose needed for oral administration is large, which further complicates the therapeutic applications of this interesting molecule. Most of the phytomedicines suffer with the inherent problem of poor bioavailability, and clinical translation of these drugs is not achieved successfully. Further, for chemopreventive effects, the phytochemical agents need to be administered or supplemented orally for longer durations. Therefore, there is a need of alternative or novel oral delivery systems to solve the issue of poor oral bioavailability and high dosage problems. In recent years, various novel pharmaceutical approaches have been utilized to improve the efficacy of Honokiol [22]. For example, HNK nanoparticles have been employed to improve its therapeutic effects in skin upon transdermal application [23]. However, to the best of our knowledge, very few attempts have been made to improve the oral bioavailability of this promising multi-pharmacological agent. The current study was designed to develop a novel Vitamin E TPGS based Honokiol loaded nanomicellar (HNK-NM) formulation and evaluate the anti-cancer effects in orthotopic TNBC mouse models. Initially, a rapid and sensitive HPLC method was developed to quantify the low levels of HNK in plasma after oral administration. Further, HNK-NM formulations were prepared and evaluated for oral bioavailability and anticancer effects were evaluated in TNBC animal models. We hypothesize that HNK formulated in nanomicellar form would increase the water solubility, which will in turn result in improved oral bioavailability and anticancer effects.

2. Materials and methods

HNK was purchased from AK Scientific, Vitamin E TPGS was generous gift sample from Antares health products, Inc (IL60174), Sterile 24 and 96 well cell culture plates and 24 well transwell inserts were purchased from Corning, USA. Cleaved Caspase 3, Cyclin D1, PCNA, Ki-67, CD31 and β-actin antibodies were procured from Cell signaling technology and Santa Cruz Biotechnology Inc, USA. BCA Protein Assay Kit was purchased from Pierce™. VEGF kit was purchased from Thermo Fisher scientific Inc, USA. Human TNBC cell lines (MDA-MB-231, 453 and 468) and Caco-2 cell lines were procured from ATCC, USA. All other chemicals used were of research grade quality.

2.1. *In vitro* anticancer effects of Honokiol on TNBC cells

In vitro anticancer assays such as cytotoxicity, clonogenic and cell migration (gap closure) assays were performed on MDA-MB-231, MDA-MB-453 and MDA-MB-468 TNBC cell lines [24]. In cytotoxicity assays, 10,000 cells/well were added to 96 wells plates and incubated overnight, thereafter cells were treated with different concentrations of HNK dissolved in dimethylsulphoxide (DMSO). Since Honokiol is not soluble in aqueous solvents, for *in vitro* studies HNK was dissolved in DMSO. To study the possible effect of DMSO on cells, solvent (DMSO) control was used at highest concentration of <0.1%. After 72 h treatment, cells were fixed and cell viability was measured by crystal violet staining (0.05%). In clonogenic assay, MDA-MB-468 cells were used. To each well of 24 well plates, 100 cells/well were plated. After allowing the cells to adjust for 24 h, cells were treated with HNK and after 48 h, cells were washed off the drug and allowed to grow as spheroids. The MDA-MB-468 cells generally grow as nice colonies and hence were suitable for this assay. These cells were allowed to grow for 2 weeks and media was changed every alternative day. The colonies/spheroids grown in each well were fixed in glutaraldehyde and stained with crystal violet. Number of colonies was counted with an optical microscope and colony sizes were measured and representative spheroid images were captured [25].

2.2. Anti-migration assay

This assay is generally used to study the *in vitro* antimetastatic effects of drugs. In this assay MDA-MB-231 cells were plated at 50,000/well density in 24 well plates. After allowing the cells to grow to confluence, in each well at middle portion, cells were scratched using sterile micropipette tip to make a gap or injury. Wells were washed with phosphate buffered saline (PBS) to remove the scratched cells. The width of each injury was measured under microscope and corresponding images were photographed. These cells were treated with HNK to study the antimetastatic potential. After 48 h, cells were fixed and stained with crystal violet and the width of the gaps was measured and images were captured. In untreated control MDA-MB-231 cells due to their high migratory potential, move into scratched gaps and narrow down the gaps (gap closure) [26].

2.3. Honokiol nanomicellar (HNK-NM) formulation preparation

Honokiol loaded nanomicelles (HNK-NM) were prepared by using amphiphilic polymer vitamin E TPGS. The nanomicellar preparation was followed according to Yu Mi et al's thin film hydration method [27]. Required amount of vitamin E TPGS was dissolved in

dichloromethane and chloroform followed by HNK-FD was dissolved in this organic phase. By using the rotary evaporator the organic phase was removed while forming the thin film on the walls of round bottom flasks. The organic solvent was completely removed by subjecting the flask to freeze drying. After complete removal of the organic solvent, these thin films were hydrated in phosphate buffer (1X PBS pH 7.4), incubated at 37 °C for 30 min, and then sonicated for 10 min. The resultant nanomicellar formulation was filtered through 0.2 μm membrane to remove un-entrapped crystalline free drug and used for the further studies. The details of polymer and drug compositions are mentioned in Table 1. Nanomicellar formulations were characterized for their size and surface charge by using Malvern zeta meter (Malvern instruments, Boston MA). The drug loading and entrapment efficiency was done by estimating entrapped drug using a well established HPLC method. The prepared final formulation was used for cytotoxic evaluation and results were compared with HNK free drug. Further, blank NM were also evaluated for cytotoxic effects.

2.4. *In vitro* drug release study

The *in vitro* release of HNK-NM was performed in PBS at pH 5.8. 1 mL of the HNK-NM was filled in nitrocellulose membrane and kept in a beaker containing 10 mL of PBS and kept on shaking water bath at 50 rpm and 37 °C temperature. At different release time points, samples were withdrawn from the beaker and same volume was replaced with fresh PBS. HNK released from nanoformulation was analyzed by an established HPLC method [28]. The cumulative drug release was calculated and percentage drug release was plotted against time. For the comparison, same amount of free drug was loaded in the nitrocellulose membrane and release profile was studied. Coumarin-6 loaded micelles were also prepared by similar method by replacing HNK with the fluorescent dye.

2.5. *In vitro* permeability and uptake studies

Caco-2 cell lines were used for *in vitro* permeability studies using our established methods [4,29]. After growing the cells as per the standard protocols, the permeability of Honokiol in free drug and nanomicellar forms were evaluated at pH 5.8. Caco-2 cell monolayers in the donor compartment were incubated with various formulations for 120 min with continuous agitation at 37 °C. In the receiver compartment, samples were withdrawn and drug concentration was estimated by HPLC. Transepithelial electrical resistance (TEER) values were measured before performing and after completion of the permeability study to confirm the integrity of the Caco-2 cell monolayers. In another set of experiments, after growing the cells in cell culture plate wells, cells were incubated with Coumarin-6 loaded nanomicellar formulation, after 2 h of incubation, cells were washed and fluorescent images were taken. Similarly, TNBC cells were also incubated with nanomicellar formulation and uptake was studied by fluorescent microscopy.

2.6. HPLC method for Honokiol

A sensitive, rapid, bioanalytical HPLC method was developed using 10 mM phosphate buffer (pH 4.5). The final composition of the mobile phase was phosphate buffer and methanol (80:20) at a flow rate of 1 mL/min. The wavelength for UV detection was set at 225 nm. A symmetry C18 column (250 mm × 4.5 mm, 5 μm) was used to separate the analytes. The retention time for HNK was 7.2 min and for internal standard (Paclitaxel) it

was 9 min. HNK and internal standard Paclitaxel (PTX) were extracted from mice plasma using simple protein precipitation. The coefficients of variation for inter-day and intra-day assay were found to be less than 10%. The mean recovery of HNK from mice plasma was found to be 98%. The limit of detection (LOD) and the limit of quantification (LOQ) of HNK-FD were determined to be 2 ng/mL and 15 ng/mL, respectively.

2.7. Pharmacokinetic study of Honokiol nanomicellar formulations

The male C57BL/6 mice were used for performing oral bioavailability studies. Our initial pharmacokinetic studies with free HNK suggested its poor bioavailability. After preparing the HNK-NM, detailed oral pharmacokinetic study was performed. Honokiol in the form of free drug and nanomicellar formulation at two dose levels (40 and 80 mg/kg) were studied for oral pharmacokinetics analysis. Mice were randomly divided into four groups and administered with free drug and nanomicellar formulations. Following the drug administration, blood samples were collected at different time points (15, 30 min, 1, 2, 4, 6, 8, 12, 18 and 24 h) by cardiac puncture into microvet collection tubes. For each sampling time point separate set (n = 3–4) of animals were used. Blood samples were immediately centrifuged at 10,000 rpm for 10 min and plasma samples were isolated and stored at −80 °C until analysis. The plasma samples were extracted for the drug by protein precipitation method and the amount of HNK present in samples was quantified by HPLC. The details of the PK data analysis was performed according to our established procedures [24,30]. The pharmacokinetic parameters such as area under the curve (AUC), C_{max}, $t_{1/2}$, t_{max}, MRT, etc were analyzed. The final formulation with improved oral bioavailability seen in PK studies was used for anticancer evaluations.

2.8. Anticancer effects of Honokiol nanomicellar formulation in orthotopic breast cancer xenograft models

Our *in vitro* assays suggested that HNK has cytotoxic effects on TNBC cell lines (MDA-MB-231 and MDA-MB-468). Based on our preliminary studies, it was observed that MDA-MB-231 cells develop perfect *in vivo* tumor models in nude mice. For anticancer in vivo studies, the MDA-MB-231 cells (2 million) were injected into mammary fat tissue. Two weeks after the tumor cell injections, palpable tumors were observed in mammary tissues, which is an indication of tumor formation. Then drug treatment either in free form or in nanomicellar forms was given orally at the dose of 40 and 80 mg/kg daily. The drug treatment was continued for 4 weeks, and the tumor volumes and body weights were recorded weekly. After 4 weeks of treatment, animals were sacrificed; final tumor volumes and weights were measured. These tumors were used for western blot and immunohistochemical analysis. For western blot experiments, tumor tissues were stored at −80 °C till the analysis was done. For IHC, tumors were fixed in formal saline [31].

2.9. Western blot analysis of tumor samples

The detailed procedure involved in western blotting analysis was performed according to our previous reports [31,32]. The protein samples were prepared by homogenizing the tumor tissues in RIPA lysis buffer containing protease inhibitors cocktail and PMSF. To load equal protein for SDS PAGE, protein quantification in extracted samples was carried by BCA method. After SDS PAGE, separated protein bands were transferred to nitrocellulose

membranes. After transfer, the membranes were incubated with primary antibodies of cleaved caspase 3, Cyclin D1, PCNA, and β-actin at 1:1000 dilution. This was followed by incubation with secondary antibodies and exposure to autoradiography films. The developed bands were quantified by densitometric analysis using Bio-Rad Chemi-Doc Image software.

2.10. Immunohistochemistry (IHC) of tumors

IHC was performed on tumor tissue sections to study the expression of tumor related markers. We have chosen CD31 and Ki-67 markers for this experiment. The TNBC tumors collected from different treatment groups were fixed in formal saline and processed according to the standard histopathological tissue processing protocol. Tumor sections were embedded in paraffin wax and 5–10 μm sections were made by using microtome. The tissue sections were placed on the poly-D lysine coated microscopic slides and used for the IHC evaluation [31]. Antigen retrieval was done in 0.01 M sodium citrate buffer (pH 6) by heating at 95 °C for 10 min followed by 30 min cooling. After antigen retrieval, the tumor sections were incubated at 4 °C overnight with selected primary antibodies Ki-67 and CD31 then incubation with biotinylated secondary antibody followed by streptavidin. The detailed procedure for IHC analysis was followed according to our previous reports [31]. The expression of Ki-67 and CD31 was evaluated by existence of the brown cytoplasmic staining. The quantification of positive cells was done by counting the brown colored cells in 10 different fields from each section. The micro vessel density (MVD) was calculated based on the CD31 positive cells.

2.11. VEGF levels in tumor lysates

The tumor tissues were homogenized in RIPA lysis buffer with protease inhibitor cocktail. The resultant tissue lysates were centrifuged and the supernatants were used for VEGF estimations by ELISA method according to the recommended protocol [31]. The protein concentrations in the supernatants were estimated by BCA method. Results were expressed as pg/mg protein. From each group 3–4 tumor samples were used and analyzed in duplicates.

2.12. Statistical analysis

The data was represented as mean ± SEM (standard error of mean). The statistical analysis was performed by using one way Anova followed by Tukey's multiple comparisons. For *in vitro* studies, 3–4 replicates were used for each time and three individual experiments were performed and results were averaged. For *in vivo* experiments n = 6–8 was used.

3. Results

3.1. Anticancer Effect of Honokiol on TNBC cells

The *in vitro* cytotoxicity assays performed with TNBC cells lines demonstrated considerable anticancer effect of HNK. Briefly, 72 h after the drug treatment, the IC_{50} values with MDA-MB-231, MDA-MB-468, and MDA-MB-453 cell lines was found to be 16.99 ± 1.28 μM, 15.94 ± 2.35 μM and 20.11 ± 3.13 μM respectively (Fig. 1A). In the clonogenic assay, HNK at 3 and 10 μM concentrations produced significant inhibition on the spheroid number and spheroid sizes (Fig. 1B and C). Compared to untreated normal control cells, HNK at 3 and

10 μM concentrations produced 1.66 and 3.72-fold significant decrease in the spheroid number; the spheroid sizes were found to be 1.99 and 9.57-fold decreased significantly with HNK treatment at 3 and 10 μM concentrations (Fig. 1B and C). The gap closure in wound healing assay indicated that HNK produced anti-metastatic effect. Further, at 3 and 10 μM concentrations, HNK produced 1.93 and 2.36-fold inhibition on the migratory potential of MDA-MB-231 cells (Fig. 1D). Fig. 2A shows the representative microscopic images of MDA-MB-231 cells after treatment with HNK for 72 h. Fig. 2B shows the representative spheroids after treatment with HNK. Fig. 2C shows the representative wound healing assays images at 0 h and 48 h after the HNK treatment which proved effective anti-metastatic potential of the nanomicellar formulation. The cytotoxic effect of HNK on normal breast epithelial cells (MCF-10A) was evaluated to understand the selectivity of this compound towards cancer cells. Interestingly, HNK was found to be relatively less toxic to normal cells, the IC_{50} value of HNK was found to be 43.02 μM (Supplementary Fig. 2). When compared to MDA-MB-468 cells, HNK produced 3-times lesser cytotoxic effects on MCF-10A cells. When we compared the cytotoxic effect of HNK in free drug form and in NM form, it was found that Honokiol in NM form produced increased cytotoxic effect compared free drug with IC_{50} value of 6.53 μM. There was approximately doubled cytotoxic effect in NM form compared to free drug form on MDA-MB-468 (Supplementary Fig. 2 A and B). Whereas, blank NM formulations even up to 100 μM concentrations also did not produce any cytotoxic effect on MDA-MB-468 cells. The IC_{50} value of blank Vitamin E TPGS NM was found to be 153.72 μM (Supplementary Fig. 2C). Further we compared the cytotoxic effect of HNK-NM on MCF-10A cells; it was observed that on normal breast epithelial cells also HNK in NM form produced relatively lesser toxic effect with IC_{50} value of 30.86 μM. Upon comparison, HNK-NM form resulted in selective cytotoxic effect on TNBC cells compared to normal breast epithelial cells with almost 4.72 fold difference in cytotoxicity, which suggest the non-cytotoxic effect of HNK-NM on normal breast epithelial cells (Supplementary Fig. 3D).

3.2. Preparation and characterization of Honokiol loaded nanomicellar formulations

HNK-NM formulation prepared with Vitamin E TPGS resulted in 30–40 nm size micelles. The entrapment efficiency of the drug was varied depending upon the percent of drug loading. The entrapment efficiency of HNK with optimized drug loading was found to be 91.5%. The basis for this selected stoichiometry of drug and Vitamin E TPGS is based on our preliminary studies and previous reports. Among the three formulations prepared, the one which has shown higher entrapment efficiency with required particle characteristics was used for further studies. The formulation details are shown in Table 1.

3.3. *In vitro* drug release profile of Honokiol nanomicellar formulations

The *in vitro* drug release of HNK-NM formulation was studied in PBS at pH 5.8. The nanomicellar formulation exhibited initial burst release of approximately 20–25% followed by sustained release up to 48 h. At the end of the release study, approximately 85% of the drug was released from the nanomicellar formulation whereas in case of HNK-FD group, only 25% of the drug was released (Fig. 3A). This release study suggested that increased solubility of HNK in nanomicellar form resulted in significant increase in the drug release ($p < 0.001$).

3.4. Caco-2 permeability and MDA-MB-468 cell uptake of Honokiol nanomicelles

The Caco-2 cell based *in vitro* permeability study indicated that apparent apical to basolateral permeability of HNK across Caco-2 monolayer was found to be 0.36×10^{-6} cm/sec. In HNK-NM formulation, this permeability was found to be increased to 1.34×10^{-6} cm/s. HNK-NM formulation produced 3.62-fold significant increase ($p < 0.001$) in the permeability of drug compared to free drug (Fig. 3B). This increase suggests that the permeability of HNK across the intestinal pathways may be enhanced with the nanomicellar formulation. The treatment of HNK either in free drug form or in nanomicellar form did not induce any alteration in TEER value which is indicative of integrity of the Caco-2 monolayer. Fig. 3C shows the representative image of Caco-2 cell uptake of Coumarin-6 loaded nanomicellar formulations. It is clearly evident from this uptake study that nanomicellar formulations significantly increased the uptake into Caco-2 cells, which is the reason why nanomicellar formulation might have produced increased permeability across the Caco-2 monolayers. Fig. 3D shows the representative uptake image of Coumarin-6 loaded nanomicellar formulations with MDA-MB-468 cell line suggesting that prepared nanomicellar formulations are readily taken up into TNBC cells. When we compared the cytotoxic effect of HNK in free drug form and in NM form, it was found that Honokiol in NM form produced increased cytotoxic effect compared free drug with IC_{50} value of 6.53 μM. There was approximately doubled cytotoxic effect in NM form compared to free drug form on MDA-MB-468 (Supplementary Fig. 2A and B). Whereas, blank NM formulations even up to 100 μM concentrations also did not produce any cytotoxic effect on MDA-MB-468 cells. The IC_{50} value of blank Vitamin E TPGS NM was found to be 153.72 μM (Supplementary Fig. 2C). Further we compared the cytotoxic effect of HNK-NM on MCF-10A cells; it was observed that on normal breast epithelial cells also HNK in NM form produced relatively lesser toxic effect with IC_{50} value of 30.86 μM. Upon comparison, HNK-NM form resulted in selective cytotoxic effect on TNBC cells compared to normal breast epithelial cells with almost 4.72 fold difference in cytotoxicity, which suggest the non-cytotoxic effect of HNK-NM on normal breast epithelial cells (Supplementary Fig. 2 D).

3.5. Honokiol HPLC method development and pharmacokinetic evaluation of Honokiol nanomicellar formulation

HNK was extracted from plasma by protein precipitation method and extraction efficiency was found to be 98%. By using the PBS:Methanol (80:20) as mobile phase, HNK was separated and retention time was found to be 7.2 min. PTX was used as internal standard and its retention time was found to be 9 min. Fig. 4A shows the representative chromatographic images of samples from blank plasma, HNK extracted plasma, PTX extracted plasma and HNK plus PTX co-extracted plasma samples. The clear-cut separation of HNK and PTX suggest the suitability of our method to analyze HNK from plasma and other biological samples (Fig. 4A).

The pharmacokinetic study of HNK-NM formulation was performed in C57BL/6 mice at the dose of 40 and 80 mg/kg/orally. HNK-NM produced significantly improved oral pharmacokinetic profile compared to free drug (Fig. 4B). The C_{max}, AUC levels, and $t_{1/2}$ value of HNK was increased in nanomicellar formulation groups compared to free drug

groups. The C_{max} levels were found to be 4.06 and 3.60-fold significantly ($p < 0.001$) increased in HNK-NM formulation groups compared to corresponding HNK-FD groups at 40 and 80 mg/kg doses (Fig. 4B and C). Compared to HNK-FD 80 mg/kg treated group, HNK-NM 40 mg/kg treated group produced 1.96-fold increase in the C_{max} levels. In terms of C_{max} levels, 40 mg/kg dose of HNK-NM formulation produced approximately 2-fold increase compared to free drug 80 mg/kg dose, suggesting that by reducing the dose to 1/4th the same plasma concentrations can be achieved by using nanomicellar formulations. The AUC levels were found to be significantly higher ($p < 0.001$) compared to free drug treated groups. At 40 mg/kg dose, HNK-NM produced 6.26-fold significant increase ($p < 0.001$) in the AUC levels compared to corresponding free drug treated groups whereas 80 mg/kg HNK-NM produced 5.83-fold improvement in the AUC levels compared to same dose of HNK-FD group (Fig. 4B and C and Supplementary Table 1). Further, at 40 mg/kg dose, nanomicellar formulation produced 3.08-fold higher plasma concentrations compared to HNK-FD at 80 mg/kg dose suggesting that by using our nanomicellar formulation approach, HNK-FD can be reduced to 1/6th of the dose required to achieve the same plasma drug levels. Therefore, by using the nanomicellar approaches the oral bioavailability of HNK can be significantly increased. On the other hand, the high oral doses required to achieve the therapeutic plasma levels can be addressed by reducing the doses in nanomicellar forms. The plasma half life ($t_{1/2}$) levels were also slightly increased in nanomicellar formulation groups compared to free drug groups at both the studied dose levels. Similarly, the mean residence time (MRT) also improved in nanomicellar formulation groups compared to free drug groups. The plasma clearance of HNK was significantly decreased in HNK-NM groups compared to free drug groups (Table 1). Overall nanomicellar formulations at both the doses exhibited significantly improved and superior oral bioavailability profile.

3.6. Anticancer effects of Honokiol nanomicellar formulations in MDA-MB-231 orthotopic xenograft models

TNBC cells (MDA-MB-231) were xenotransplanted in nude mice(orthotopic model in mammary fat pads) and treated with HNK-FD and HNK-NM formulations at the dose of 40 and 80 mg/kg orally. Treatment was given daily for 4 weeks and during this period tumor volumes were monitored weekly. Both doses of HNK-FD and HNK-NM formulations produced significant anticancer effects. Two weeks after the treatment, significant decrease in the tumor volumes were observed in all the treatment groups. Further, this decrease in the tumor volume was more significant at 3 and 4 weeks post treatment. At 40 mg/kg dose, HNK-NM produced 1.78-fold superior reduction in the tumor volumes compared to free drug treated groups. Similarly, at 80 mg/kg dose, HNK-NM produced 2.08-fold superior anticancer effects. HNK-NM at 40 mg/kg produced 1.36-fold superior anticancer effects compared to HNK-FD 80 mg/kg dose, which means even by reducing the dose to half, superior anticancer effects are observed in nanomicellar formulation treated groups (Fig. 5A and B). Similarly, at the end of the treatment, tumor weights were also significantly decreased in nanomicellar formulation treated groups compared to respective free drug treated animals. At 40 and 80 mg/kg doses of HNK-NM, formulation treated groups produced 2.19 and 2.29-fold significant reduction in the tumor weights compared to HNK-FD treated animals. Moreover, 40 mg/kg dose of HNK-NM exhibited 1.54-fold superior reduction in the tumor weights compared to 80 mg/kg dose of HNK-FD treated groups (Fig.

5C). Both tumor volumes and tumor weights demonstrate that HNK-FD in nanomicellar form exhibit superior anticancer effects compared to free drug treated groups. The representative orthotopic tumor bearing mice and isolated tumors from control, HNK-FD 80 mg/kg and HNK-NM formulation 40 mg/kg treated groups are shown in Fig. 5D.

3.7. Effect of Honokiol nanomicellar formulations on proliferation and Apoptosis

The western blot based analysis of these three markers suggested the superior anticancer effects of nanomicellar formulations. The cell proliferation marker PCNA was found to be 1.38 and 2.66-fold significantly decreased in HNK-FD and HNK-NM treated groups, respectively compared to untreated control tumors (Fig. 6A and C). A 1.80 and 3.99-fold decrease in the cell cycle regulating kinase cyclin D1 expression was observed in free drug and nanomicellar treated groups respectively, compared to control tumors (Fig. 6A and B). The proapoptotic marker cleaved caspase 3 was found to be 3.51 and 5.91-fold significantly increased in HNK-FD and HNK-NM formulation treated tumors, respectively compared to control tumors, which suggest the role of apoptosis in HNK mediated anticancer effects (Fig. 6A and D). In all the three markers studied by western blot analysis, HNK-NM produced superior anticancer effects compared to HNK-FD treated groups. The PCNA, Cyclin D1 and cleaved caspase 3 expressions were found to be 2.12, 1.92 and 1.68-fold significantly altered in 40 mg/kg HNK-NM formulation treated groups compared to 80 mg/kg dose HNK-FD treated groups, which confirms the enhanced anticancer effect of nanomicellar formulation in TNBC tumor model (Fig. 6).

3.8. Effect of Honokiol nanomicellar formulation on expression of Ki-67 and CD31

The immunohistochemical analysis of cell proliferation marker Ki-67 and tumor angiogenic marker CD31 also demonstrated the superior anticancer effects of HNK-NM. Ki-67 indicates the nature of the cell proliferation potential of tumors, its expression was found to be relatively higher in untreated control tumors, where as treatment with HNK-FD and HNK-NM form resulted in marked decrease in the expression of Ki-67 (Fig. 7A and B). The quantitative analysis of Ki-67 expressing cells indicated a 1.57 and 2.73-fold ($p < 0.05$ and 0.01) decrease in 80 mg/kg HNK-FD and 40 mg/kg HNK-NM formulations treated groups respectively, compared to control tumors. The CD31 expression indicates the tumor vascular status and it is well documented that due to increased angiogenesis, the expression of CD31 is increased in aggressive tumors like TNBC [33]. Based on the CD31 expression, the mean vascular density (MVD) was calculated. There was a significant reduction in the expression of MVD in HNK-FD treated groups. It was observed that a 1.36 and 2.09 fold decrease in the MVD expression was observed in HNK-FD and HNK-NM formulation treated groups, respectively (Fig. 7A and C). Both the markers studied by IHC analysis demonstrated that 40 mg/kg dose of HNK-NM formulation exhibited 1.73 and 1.53 fold significant reduction in the Ki-67 and MVD expressions respectively, compared to 80 mg/kg dose of HNK-FD treated groups (Fig. 7).

3.9. Effect of Honokiol on VEGF levels and body weight changes

The estimation of VEGF levels in tumor lysates and plasma indicates the vascular status of tumors. Plasma VEGF levels were found to be significantly decreased in HNK-FD and HNK-NM formulation treated groups. The tumor VEGF levels indicate more relevant

information on tumor vasculature, and these levels were found to be 1.55 and 2.88-fold significantly decreased in 80 mg/kg HNK-FD and 40 mg/kg HNK-NM formulation treated groups, respectively. VEGF levels were found to be 1.39 and 1.85-fold decreased in 40 mg/kg HNK-NM formulation compared to 80 mg/kg free drug treated groups, which suggest the superior antiangiogenic effects of HNK-NM formulation compared to free drug (Supplementary Fig. 1A). The body weight changes were monitored throughout the treatment period and it was found that there was no significant change in HNK-FD and HNK-NM formulation treated groups, which suggests the safety profile of HNK-FD at as high as 80 mg/kg oral dose and also ensured the safety of HNK-NM formulation (Supplementary Fig. 1B).

4. Discussion

HNK shows potential multiple pharmacological effects in several preclinical models, but its poor water solubility significantly restricts clinical applications. HNK, owing to its high lipophilicity cannot be dispersed in water, which makes oral and intravenous administration difficult [34,35]. Honokiol has already been prepared as nanoformulation co-loaded with sirolumus to increased oral bioavailability of HNK by its P-gp inhibiting property [36,37]. Though HNK nanoformulations are available, very few of them have demonstrated increased oral bioavailability. There is an increased need to develop suitable novel formulations for oral administration. In a recent study, the role of nanosuspension based Honokiol formulation to improve the oral bioavailability and increased concentration of Honokiol in cardiovascular and cerebrovascular system has been demonstrated [35]. Further, the intraperitoneal administration of Honokiol nanosuspension produced dramatic increase in the drug concentrations in targeted tissues; however, for the required selective distribution of Honokiol the formulation has to be administered by non-oral routes. Several approaches have been attempted to increase the aqueous solubility of HNK. Monomethoxy poly(ethylene glycol)-poly(lactic acid) (MPEG-PLA) was used to formulate HNK loaded nanoparticles, which produced increased solubility [34]. For intravenous administration, HNK-FD was encapsulated in PEG-PCL-PEG [38]. In another study, loading of HNK into self assembled pectin nanoparticles was carried out to deliver to HepG2 cells and it was observed that the anticancer activity was increased in the nanoformulation group compared to free drug [39]. Though, nanoparticle based HNK formulations have been reported, most of the studies suggest that these formulations are suitable for intravenous administration. There is an increased need to develop suitable novel formulations for oral administration. The application of nanomicellar system to increase the solubility and permeability was observed by using Pluronic F127 copolymer [23] which suggests that by addressing the rate-limiting step of poor aqueous solubility, the desired oral bioavailability and pharmacological activity can be achieved. So far, most of the *in vivo* studies reported with HNK were based on either parenteral route of administration or high doses of oral administration [40]. However, it is quite impossible to achieve pharmacologically active doses in humans while translating the animal studies into clinical use. Therefore, there is a need to address the inherent biopharmaceutical problems associated with potentially safer phytopharmaceuticals like HNK. In the current study, an attempt was made to prepare the nanomicellar formulation of HNK by using vitamin E TPGS. The prepared nanomicellar formulation was

in the size range of 20–40 nm, by virtue of such small size, prepared formulations produced increased permeability across Caco-2 monolayers, and the oral bioavailability was found to be greatly increased in nanomicellar formulations. The possible explanation for this increase in permeability is the increased solubility of HNK in nanomicellar formulation and increased uptake of Caco-2 cells *in vitro* and intestinal epithelial enterocytes *in vivo* [41,42]. The Caco-2 cell uptake study confirmed this effect. In the oral pharmacokinetic study, HNK-NM formulation produced greater increase in the oral bioavailability profile. The C_{max} and AUC levels were found to be significantly increased in both doses of HNK-NM compared to respective free drug groups. The C_{max} levels at 40 and 80 mg/kg in HNK-NM were 4.06 and 3.60-fold, respectively increased compared to free drug treated groups. Interestingly, the C_{max} levels in HNK-NM 40 mg/kg groups were found to be 1.96-fold higher than 80 mg/kg of free drug treated group indicating that 4-fold dose reduction is possible with our prepared nanomicellar formulation compared to HNK-FD treatments. Similarly, the AUC levels were also 6.26 and 5.83-fold increased in HNK-NM formulation group compared to respective free drug groups of 40 and 80 mg/kg. The AUC levels in 40 mg/kg HNK-NM group was 3.08-fold greater than the 80 mg/kg group suggesting that approximate 6-fold reduction in the oral dose is possible with nanomicellar approaches. This type of favorable oral pharmacokinetic profile is quite remarkable and this approach may solve the high dose problems associated with promising natural products for various medicinal applications. Our results are not surprising, because many studies demonstrated the significant increase of oral bioavailability of several natural products when delivered as nanoformulations [30,43–46].

Development of novel therapy for treating TNBC is one of the unmet needs due to various associated problems. On the other hand, natural product based anticancer drugs exhibit better safety profile compared to standard anticancer drugs [24,47–50]. Our *in vitro* studies demonstrated the potential anticancer effects of HNK on different TNBC cells lines. Further, the cytotoxic effects on normal breast cell line (MCF-10A) did not result in any toxic effect, which suggest that Honokiol produces selective anticancer effects on cancer cells while sparing the normal breast cells. After achieving the significant increase in the oral bioavailability with nanomicellar formulations, we were motivated to evaluate the therapeutic efficacy in orthotopic TNBC models. Initially, we have confirmed the cytotoxic effect of HNK-NM on MDA-MB-468 cells and found that HNK in NM form produced significant increase in the *in vitro* anticancer effects. The possible reason for this effect is increased uptake of HNK in NM form. Though, HNK produced significant anticancer effect in xenograft models, these effects were seen at 40 and 80 mg/kg doses, which is considerably high. Translating these active preclinical doses for clinical use, very high doses of free drug is required whereas in nanomicellar groups; same doses produced superior anticancer effects. At 40 and 80 mg/kg doses, HNK-NM formulations produced 1.78 and 2.08 fold superior anticancer effects compared to HNK-FD treated groups. The increased anticancer activity is possible because of the significant improvement in the oral absorption of HNK-FD in nanomicellar formulations. It is surprising to note that out of eight animals treated with HNK-NM 80 mg/kg doses, two of the animals (25%) exhibited complete tumor regression. The HNK-NM formulation at lower dose of 40 mg/kg also produced 1.36 fold superior anticancer effects than HNK-FD 80 mg/kg treated animals. Similarly, the tumor weights were also significantly decreased in HNK-NM formulation treated animals

compared to respective free drug treated animals. Honokiol has been shown to inhibit the growth of both estrogen receptor-positive (MCF-7) and -negative breast cancer cell lines (MDA-MB-231) in G1 phase of cell cycle and by induction of caspases; indicating its efficacy in both subtypes of breast cancer [14]. Similar findings on G1 phase inhibition and caspase dependent apoptosis induction by Honokiol were reported in case of colorectal and pancreatic cancer [51,52]. However, TNBC is more aggressive form of breast cancer and needs superior biological efficacy and using nanoplatform may cater this need. The western blot analysis of tumor samples also demonstrated superior anticancer effects of HNK-NM formulation. At the molecular level, cyclin D1 plays an important role in the proliferation of cancer cells. HNK-NM treatment significantly down regulated the expression of this cell cycle related marker. The effect of HNK on cyclin dependant cell cycle process is reported in pancreatic cancer and our *in vivo* studies are in agreement with those studies [51]. The cell proliferation marker PCNA was significantly down regulated in HNK-FD treated tumors; further, nanomicellar formulation exhibited superior down regulation of PCNA expression compared to free drug treated tumor samples. The apoptotic marker, cleaved caspase 3 expression was significantly increased in HNK-FD groups and nanoformulations produced superior induction of apoptosis compared to free drug treated groups. In all the western blot markers studied, HNK-NM 40 mg/kg formulation exhibited significantly increased anticancer effects compared to 80 mg/kg free drug treated groups. The western blot analysis supported the superior *in vivo* anticancer effects of HNK-NM formulations. The superior anticancer effects with HNK-NM formulation are possible because of the increased oral bioavailability of HNK in nanomicellar forms. Such kind of superior pharmacodynamic effects are demonstrated with several nanoformulation approaches. However, to the best of our knowledge this is the first study to demonstrate the increased oral bioavailability and anticancer activity of HNK in nanomicellar formulation. This formulation approach has significant potential to develop novel oral drug delivery approaches for poorly bioavailable anticancer drugs from natural sources. The increased anticancer effects observed with HNK-FD formulation were also further confirmed from the IHC analysis of tumor sections. The proliferation marker Ki67 expression was significantly decreased in nanoformulation treated groups compared to free drug treated groups. The increased expression of cleaved caspase 3 observed in nanomicellar formulation group suggest the role of apoptosis in HNK mediated anticancer effect. Moreover, this increased apoptosis is most likely possible due to increased bioavailability of HNK in nanomicellar formulation. The decreased expression of CD31 in Honokiol treated groups suggests the effect of this drug on angiogenesis. The corresponding MVD which is an indicator of angioneogenesis is significantly decreased in nanomicellar formulation treated groups compared to HNK-FD treated groups. The antiangiogenic effects of HNK are also reported in other cancer models and our observations are in agreement with those reports [8]. The decreased levels of VEGF in HNK-NM formulation further confirm the antiangiogenic effect of HNK and superior antiangiogenic effects of HNK-NM formulations. Long term 4 weeks of daily treatment of HNK either in free drug or nanomicellar forms did not induce any kind of adverse effects in the animals, which suggest the desirable safety profile of HNK. The toxicity issues associated with conventional chemotherapeutic drugs limit their use in treating several cancers. Therefore, drugs like HNK having excellent safety profiles can be considered as alternative therapy to treat TNBC [13]. Further, nanomicellar

formulation approaches may increase the pharmacokinetic and pharmacodynamic performance of these safer drugs. Our nanomicellar formulation could be a very promising strategy to leverage the maximum clinical outcome upon oral administration of HNK and may take it one step closer to the clinical setting for oral dosing.

5. Conclusion

The limitation of oral bioavailability of HNK was successfully overcome by formulating it as nanomicellar formulations. Due to significant increase in oral bioavailability and superior anticancer effects, our results suggest that nanomicellar formulation is a suitable formulation approach for oral delivery of Honokiol. The use of vitamin E TPGS for nanomicellar formulation resulted in significant increase in the oral bioavailability of HNK. The increased oral absorption of HNK resulted in significant increase in anticancer effects in orthotopic triple negative breast cancer models. Our results clearly demonstrate that nanomicellar formulation is a superior alternative than other approaches for anticancer compounds like HNK. Our nanomicellar formulation approaches opens new avenues of delivery strategies to improve the therapeutic performance of orally active safe anticancer drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was supported by the NIH Minority Biomedical Research Support (MBRS)-SC1 program [Grant # SC1 GM092779-01; MS] and the National Institute of Minority Health and Health Disparities (NIMHD) NIMHD P20 [Grant # 1P20 MD006738-03; MS].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2017.01.038.

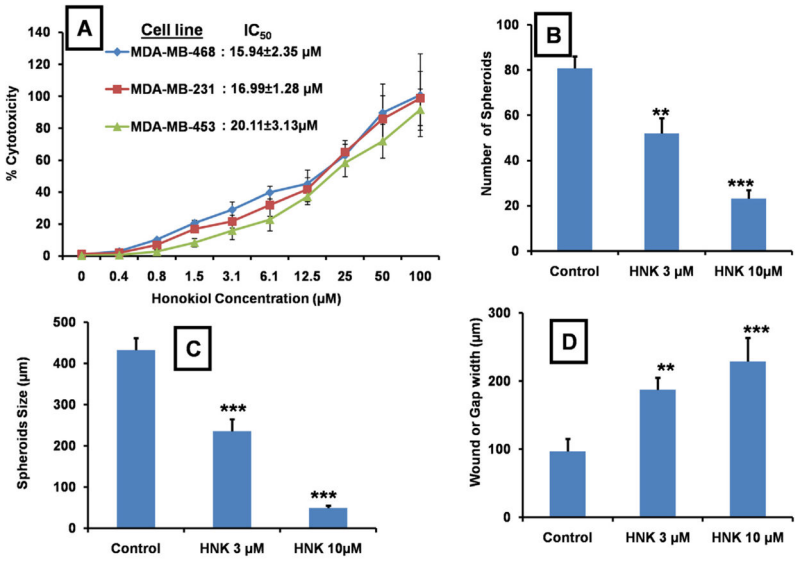

Fig. 1.

In vitro anticancer study: Cytotoxicity, Spheroids and wound healing data. A) Concentration dependant cytotoxic effects of Honokiol on MDA-MB-468, MDA-MB-231 and MDA-MB-453 cells, this figure also shows the IC_{50} values of Honokiol on all these three TNBC cells lines. B) Effect of Honokiol at 3 and 10 μM concentrations on spheroid number studied with MDA-MB-468 cells, C) Spheroid sizes. D) Effect of 3 and 10 μM Honokiol on cell migration (wound healing assay), this figure shows the wound gap widths. Each data point is represented as mean ± sem (n = 4–8). **p < 0.01 and ***p < 0.001 Vs respective control groups.

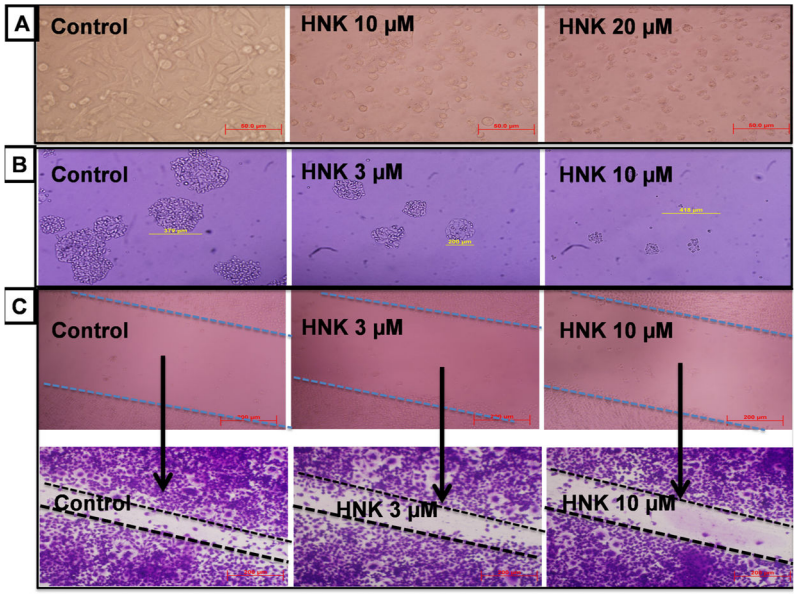

Fig. 2.

Representative images of *in vitro* studies. A) Effect of Honokiol on morphological features of MDA-MB-231 cells treated with 10 and 20 μM of Honokiol, B) Effect of Honokiol at the concentration of 3 and 10 μM on clonogenic potential of MDA-MB-468 cells, C) Effect of Honokiol on migration potential of MDA-MB-231 cells (wound/gap closure assay) images show the gap widths of control and Honokiol treated cells at staring of the wound formation (0 h) and gap widths of respective groups 48 h after Honokiol treatment, HNK: Honokiol.

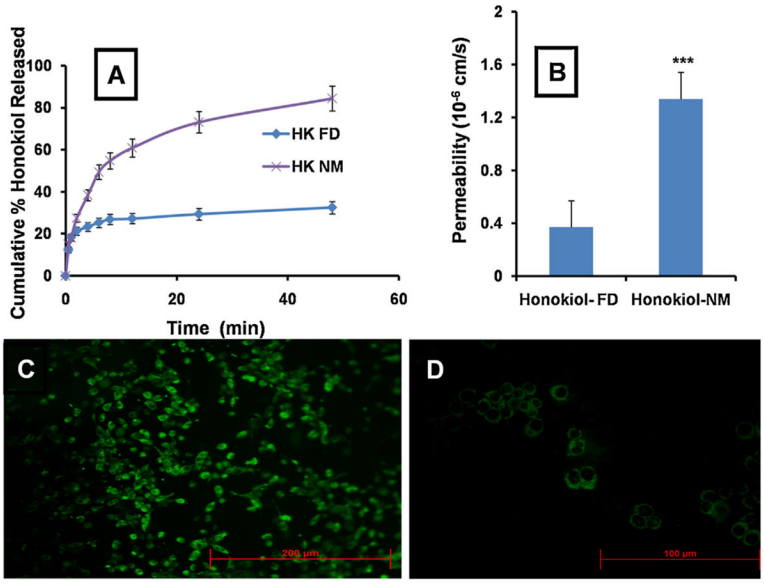

Fig. 3.

In vitro drug release, Caco-2 permeability and TNBC cell uptake studies. Figure A) *In vitro* drug release profile of selected HNK-NM, B) The Caco-2 permeability of HNK-NM, C) Representative microscopic image of Coumarin-6 loaded nanomicellar uptake into Caco-2 cells and D) Representative microscopic image of uptake of fluorescent dye loaded Vitamin E TPGS Nanomicelles into MDA-MB-468 cells 2 h after the incubation.

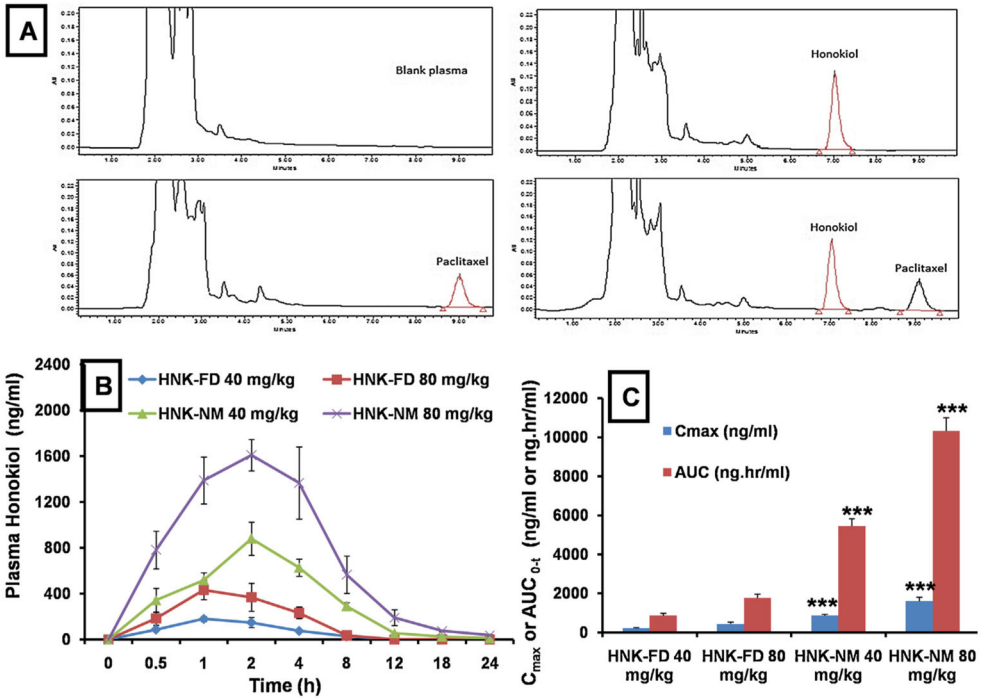

Fig. 4.

Pharmacokinetic studies: HPLC method development for analysis of Honokiol: A) Typical HPLC chromatograms of blank plasma (top left), plasma spiked with Honokiol (500 ng/mL) (top right), Plasma spiked with internal standard (Paclitaxel, 500 ng/mL) (bottom left) and chromatogram of Honokiol after oral administration (40 mg/kg) (top right) in mice plasma. B) Pharmacokinetic profile of Honokiol free drug and Honokiol NM upon oral administration at 40 and 80 mg/kg body weight dose and C) C_{max} and AUC values of Honokiol in different groups. Each data point was represented as mean ± sem (n = 3–4). ***$p < 0.001$ Vs respective dose matched HNK-FD groups.

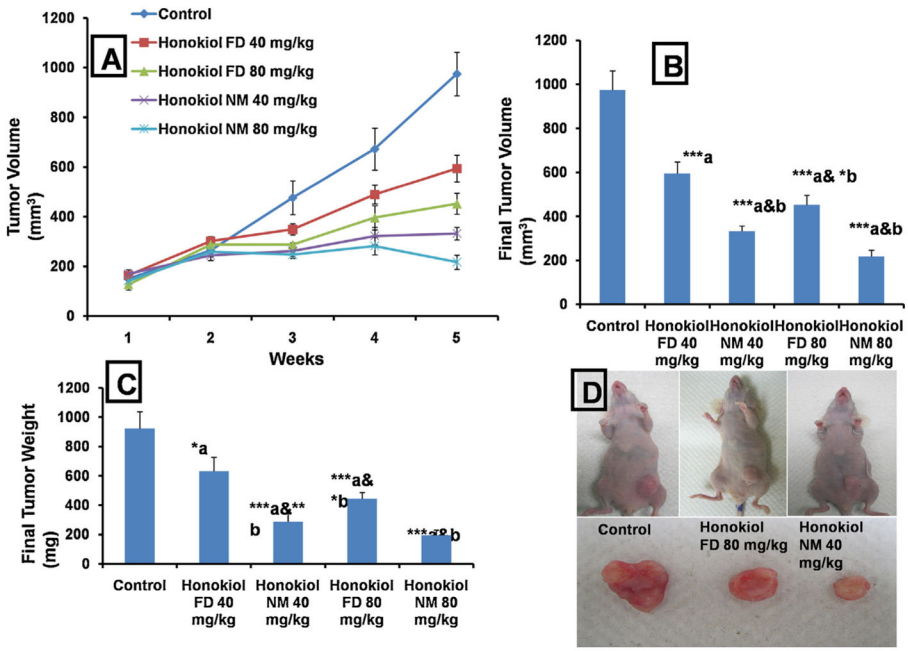

Fig. 5.

Anticancer effects of Honokiol NM formulations in orthotopic breast cancer model. A) Tumor volumes during the 4 weeks of Honokiol NM oral administration in TNBC bearing mice. B) Final tumor volumes after completion of 4 weeks of Honokiol free drug (HNK-FD) and nanomicellar (HNK-NM) formulation treatment at 40 and 80 mg/kg doses. C) Final tumor weights after completion of 4 weeks of HNK-FD and HNK-NM treatment at 40 and 80 mg/kg doses. Each data point was represented as mean ± sem (n = 6–8). *$p < 0.05$ and ***$p < 0.001$ a vs untreated control groups and b vs respective HNK-FD 40 groups. D) Representative orthotopic breast tumor bearing mice from untreated control, HNK-FD 80 mg/kg and HNK-NM 40 mg/kg, figure also shows the isolated tumors from corresponding groups. HNK-FD: Honokiol free drug and NM: Honokiol nanomicellar formulation.

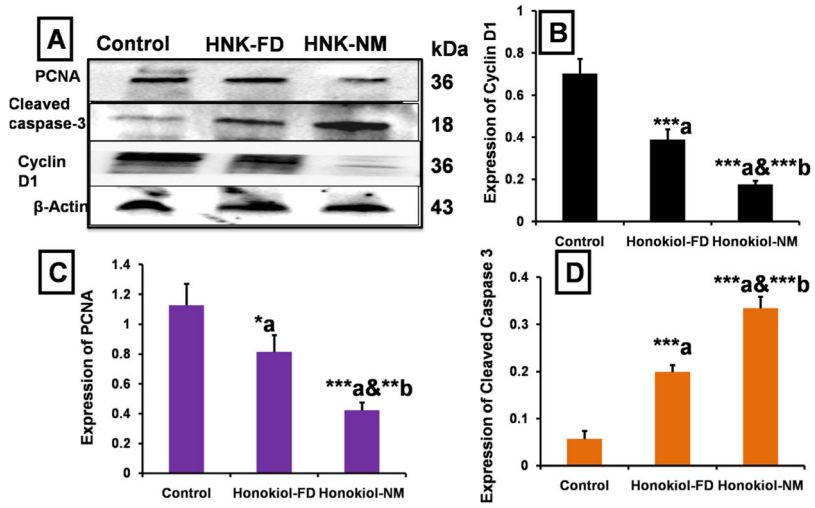

Fig. 6.

Western blot analysis: A) Representative western blot band images of PCNA, Cleaved Caspase 3, Cyclin D1 and β-Actin from control, HNK-FD 80 mg/kg and HNK-NM 40 mg/kg treated tumors. Densitometric analysis of western blot bands for quantification B) Cyclin D1, C) PCNA and D) Cleaved Caspase 3. Data was represented as mean ± sem (n = 3–4). *p < 0.05, **p < 0.01 and ***p < 0.001 a vs untreated control groups and b vs HNK-FD 80 mg/kg treated groups.

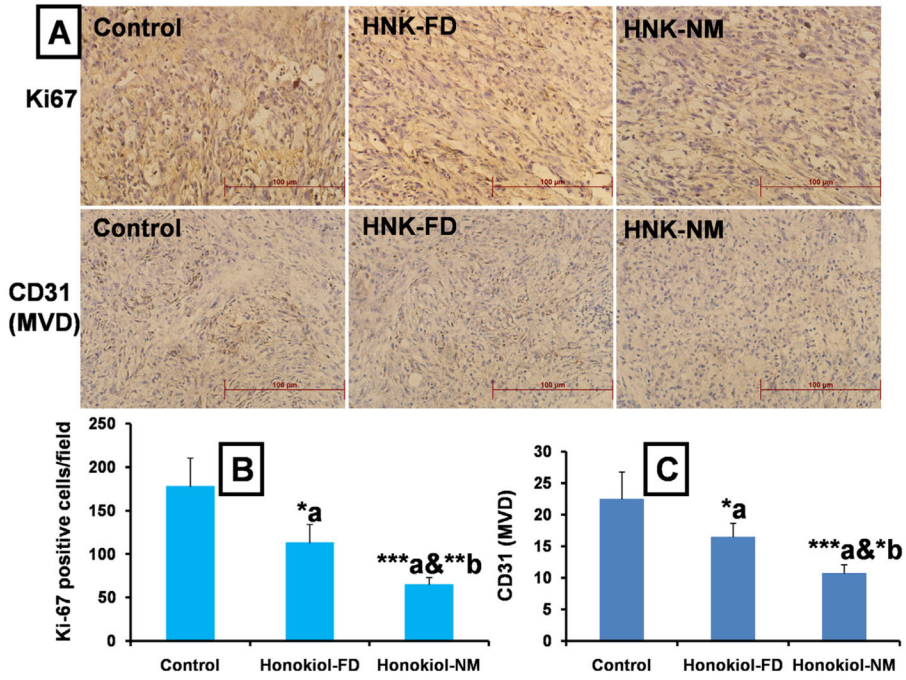

Fig. 7.

IHC analysis of tumors: A) Representative photomicrographs of IHC analysis of tumor sections from control, HNK-FD 80 mg/kg and HNK-NM 40 mg/kg treated tumors sections for cell proliferation marker Ki-67 and angiogenesis marker CD31. The CD31 expression was quantified as microvascular density (MVD). B) Quantification of Ki67 expression and C) Quantification of MVD expression in different groups. For quantification of number of IHC positive cells per filed, cells were counted from each slide at 10 different fields and for each group minimum of 3 slides were used. Data was represented as mean ± sem (n = 3–4). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ a vs untreated control groups and b vs HNK-FD 80 mg/kg treated groups.

Table 1

Optimization of Honokiol loaded Vitamin E TPGS nanomicellar formulations.

Drug (Honokiol): Polymer (Vitamin E TPGS) Ration	1:10	1:15	1:20
Entrapment Efficiency (EE)	72%	83%	91.5%
Nanomicellar Size	21 nm	32 nm	36 nm
Particle Charge (mV)	−30.23	−32.48	−34.31
Poly dispersity Index (PDI)	0.31	0.28	0.25