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Evaluation Of Synergistic In Vitro Antioxidant And Anticancer Effect Of Ethanolic Extract Of Polyherbal Mixture

Dr K Priya Gayathri[1], Dr R Kavitha[2], Dr M Najma Sherin[3]
[1]Senior Resident, Sri Ramachandra Institute Of Higher Education And Research, Porur, Chennai-600116.
[2]Professor And Head,Sri Ramachandra Institute Of Higher Education And Research, Porur, Chennai-600116.
[3]Senior Resident, Sri Ramachandra Institute Of Higher Education And Research, Porur, Chennai-600116.

Abstract
Polyherbal preparations are considered advantageous in the treatment of cancer due to presence of mixture of phytoconstituents that might have synergistic effect with less side effects. Hence in the present study, polyherbal mixture comprising of Curcuma longa (turmeric), leaf of Ocimum sanctum (tulsi), root of Glycyrrhiza glabra (Licorice), dried fruit of Piper nigrum (black pepper) and dry rhizome of Zingiber officinale (dry ginger) was prepared. The ethanolic extract of this polyherbal mixture (PHM-EE) was prepared suing Soxhlet apparatus. PHM-EE showed in vitro antioxidant activities such as DPPH radical and nitric oxide radical scavenging activities in dose-dependent manner. The IC_{50} (half-maximal inhibitory concentration) values of PHM-EE against DPPH and NO scavenging activity was found to be 16.073 µg/ml and 33.17 µg/ml, respectively. Further, PHM-EE showed anticancer activity against breast cancer cell, MCF-7. PHM-EE treatment reduced the viability of MCF-7 with IC_{50} value of 7.8 µg/ml. The PHM-EE treatment induced apoptosis in the MCF-7 cells which was visualized as DNA fragmentation in agarose gel electrophoresis. Altogether, the polyherbal preparation showed dual functional activity such as antioxidant and anticancer activity which could be further studied for its therapeutic potential.
Keywords *Anticancer, antioxidant, DNA fragmentation, Curcuma longa, Ocimum sanctum, Glycyrrhiza glabra, Piper nigrum, Zingiber officinale*

INTRODUCTION

Breast cancer is a global health issue which is the major cause of cancer death in Women. Global cancer statistics has estimated the incidence of 22.6 million cases of breast cancer and 0.68 million deaths worldwide in 2020. About 1 in 4 cancer cases and 1 in 6 cancer deaths among women accounts for breast cancer in the vast majority of countries though out the World.[1] Genetic, environmental and life style factors individually or in combination increases the incidence of cancer and risk of morbidity.[2] At present, the treatment protocol to overcome breast cancer involves multidisciplinary approach that includes various therapies such as ionizing radiation, surgical resection and medical oncology. Medical oncology encompasses immunotherapy, hormonal therapy and chemotherapy which are considered as the main treatment options for early-stage breast cancer. However, development of drug resistance, toxicity of drugs in normal cells and various side-effects has reduced the efficacy of these therapies.[3] Hence, identifying natural-based anticancer agents with low toxicity and selectivity towards cancer cells are in urge.

Traditional medicinal practices in India (Ayurveda and Siddha) and China (traditional Chinese medicine) uses numerous medicinal plants for the treatment of cancer. The in-effectiveness of chemotherapeutic agents used at present warrants the exploration into alternative compounds of natural-origin to improve today's therapy regimens or to act as a means of chemoprevention.[4] Such studies have proved beneficial with the identification of paclitaxel, an alkaloid present in the *Taxus breyifolia* (pacific yew tree).[5] Paclitaxel is used in the treatment of breast, ovarian, lung, bladder, prostate, melanoma, esophageal, as well as other types of solid tumor cancers. Following paclitaxel, docetaxel an analogue of paclitaxel, has been developed to treat breast, gastric, prostate, and head and neck cancers.[6] Another potent plant-acquired active compound, Homoharringtonine extracted from the Chinese tree *Cephalotaxus harringtonia* has been used successfully for a long time in China for the treatment of acute myelogenous leukemia. Also, vinblastine and vincristine from the Madagascar periwinkle, and *Catharanthus roseus* G. is used treat acute lymphocytic cell leukemia, Hodgkin disease and non-Hodgkin disease clinically.[7] In addition to these compounds, various other studies have identified natural-compounds such as berberine, neferine, capsaicin, curcumin, ethoxysanguinarine, piperlongumine, icariin, luteolin, naringenin, quercetin, silibinin, formononetin, epicatechin, caffeic acid, dicoumarol, resveratrol, acetogenin, prodigiosin, eudesmol, betulinic acid, ingenol mebutate, emodin, dicoumarol, bixin, indole-3-carbinol, brusatol, acetogenin and prodigiosin as an enhancer of cancer therapy.[8] Around 100 unaltered natural-compounds as well as their derivatives were involved in clinical trials against various ailments, with majority of these compounds are studied

for anticancer activity.[9] Between 1981 and 2019, it is estimated that about 25% of all newly approved anti-cancer drugs were related to natural products.[10-12]

Although natural-based compounds individually have anticancer potential, polyherbal medicinal preparations are preferred because various medicinal plants have complex mixture of bioactive phytochemicals, some provide desired therapeutic activity, while others reinforce the therapeutical potential of active principles present in the formulation and some other compounds might neutralize or counteract the possible side-effects. Hence, the synergistic effect of these natural-compounds is considered advantageous in the treatment of cancer.[13] Accordingly, previous studies have shown the anticancer effect of polyherbal formulations such as Indukantha Ghritha, Jiedu Xiaozheng, Antarth, Yin, HC9, Le Pana Guliya, Panchakola, triphala, V2S2, PHF5, 3HX and SJT ONC against various cancer cell line.[13-23] The polyherbal formulations such as Teng-Long-Bu-Zhong-Tang, Yiqi-yangyin-jiedu decoction and PHY906 are in different stages of clinical trial for anticancer therapy.[8] Importantly, the advantage of using polyherbal formulation as adjuvant therapy are seen as a synergistic effect (the retention of the same efficacy or sometimes a higher efficacy) as well as lowering of doses which could lead to lower resistance.

Keeping these views, in the present study, pharmacologically viable extract of polyherbal mixture (PHM) consisted of five medicinal ingredients such as root of *Curcuma longa* (turmeric), leaf of *Ocimum sanctum* (tulsi), root of *Glycyrrhiza glabra* (Licorice), dried fruit of *Piper nigrum* (black pepper) and dry rhizome of *Zingiber officinale* (dry ginger) was prepared. All these ingredients are used in traditional medicine for the treatment of various ailments. The prepared polyherbal mixture, PHM was extracted using the solvent ethanol in Soxhlet apparatus. The ethanol extract of the PHM (PHM-EE) was studied for its antioxidant effect in *in vitro* and anticancer effect against breast cancer cell line (MCF-7).

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), antibiotic solutions, (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and butylated hydroxytoluene (BHT) were purchased from Himedia Pvt Limited, Mumbai, India. Griess reagent was purchased from Sigma Aldrich Chemicals Co, USA. All other chemicals used were procured from Himedia Pvt Limited, Mumbai, India and of the highest purity.

Preparation of polyherbal mixture (PHM)

Individual ingredients of the polyherbal mixture such as dry turmeric root, dried leaf of tulsi, dry root of licorice, dry fruit of black pepper, dry ginger was collected from Rathinamangalam, Chennai, India. The materials were authenticated by Botanist.

All the materials were powdered. Dried powder of individual herbal materials such as turmeric, tulsi, licorice, black pepper and dry ginger were combined in fixed doses of 5g each.

Preparation of PHM-EE

About 10 g of PHM powder were extracted using 100 ml of ethanol in the ratio of 1:10 w/v by keeping in Soxhlet apparatus for 8 h. The obtained extracts were dried using rotary evaporator at reduced pressure (in vacuum at 40 ^{o}C). The dried extract was stored at 4 ^{o}C until further use. For determination of antioxidant and anticancer activity, desired concentration of extract was prepared using dimethyl sulfoxide.

In vitro antioxidant assays

DPPH scavenging activity

DPPH scavenging capacity of the PHM-EE Was estimated according to the previously reported method with slight modification using the stable DPPH radical.[24] About 9 mM of DPPH was prepared in methanol. For DPPH scavenging assay, the reaction mixture consisted of 200 µl DPPH solution, various concentrations of extract and the solution was made up to 4 ml with methanol. The reaction mixture was incubated for 30 minutes in dark at room temperature. The free radical scavenging potential of the extracts were expressed as the disappearance of the initial purple color. The absorbance of the reaction mixture was read at 517 nm. BHT was used as control.

$$\text{Inhibition activity (\%)} = \left[\frac{\text{Absorbance (Control)} - \text{Absorbance(Sample)}}{\text{Absorbance (Control)}}\right] * 100$$

Nitric oxide (NO) scavenging activity

The reaction mixture contained 10 mM sodium nitroprusside in phosphate buffer saline (pH 7.4) and various concentration of PHM-EE. The control tube was set without extract. The tubes were incubated 25 °C for 1 hour. At the end of incubation, equal volume of Griess reagent was added. A true blank was set with various concentration of plant extract in phosphate buffer saline without sodium nitroprusside and Griess reagent. The chromophore formed was measured at 546 nm against the blank.[25] Ascorbic acid was used as a standard.

$$\text{Inhibition activity (\%)} = \left[\frac{\text{Absorbance (Control)} - \text{Absorbance(Sample)}}{\text{Absorbance (Control)}}\right] * 100$$

***In vitro* anticancer activity**

Treatment of cell line with PHM-EE and cell viability assay

MCF-7 cell lines (maintained under an atmosphere of 5% CO_2 at 37 °C) were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. All the assays were carried out at 80% confluence of the cells. The MCF-7 cell line was treated with various concentrations (7.8 - 1000 µg/ml) of PHM-EE for 24 h. At the end of the treatment cell viability was determined by MTT assay. Briefly, the media was removed and 200 µl of DMEM containing 100 µg of MTT was added. The cells were incubated for 4 h at 37 °C and the medium was removed. The dark blue formazan was solubilized using 100 µl of DMSO and the blue colour formed was read at 570 nm using a micro plate spectrophotometer (BioTek Epoch2, Agilent, USA).

$$\text{Cell viability (\%)} = \left[\frac{\text{Absorbance (Sample)}}{\text{Absorbance (Control)}}\right] * 100$$

DNA fragmentation assay

The MCF-7 cells treated with PHM-EE (7.8 µg/ml) for 24 h were lysed using 500 µl of lysis buffer [10 mM tris (pH 7.4), 150 mM sodium chloride, 5 mM ethylenediaminetetraacetic acid and 0.5% triton X-100]. The lysate was centrifuged and the DNA from the supernatant was extracted using ice-cold isopropanol overnight at -20 °C as precipitate. The DNA was quantified using UV-vis spectrophotometer and equal amount of each DNA samples was run at 0.8% of agarose gel electrophoresis along with ladder.[26] The image was captured using UV-visible transilluminator.

RESULTS

***In vitro* antioxidant activity of PHM-EE**

DPPH scavenging activity of PHM-EE

The DPPH free radical scavenging activity of PHM-EE and control BHT is shown in figure 1A and 1B, respectively. PHM-EE showed dose-dependent scavenging activity similar to that of the control BHT. Also, the DPPH free radical scavenging capacity of the PHM-EE is comparable to that of the control BHT. The IC_{50} (half-maximal inhibitory concentration) value of PHM-EE for DPPH scavenging activity was found to be 16.073 µg/ml.

NO scavenging activity of PHM-EE

The NO free radical scavenging activity of PHM-EE and control BHT is shown in figure 2A and 2B, respectively. PHM-EE showed dose-dependent scavenging activity of NO similar to that of the control ascorbic acid. Also, the NO free radical scavenging capacity of the PHM-EE is comparable to that the control ascorbic acid. The IC_{50} value of PHM-EE for NO scavenging activity was found to be 33.17 µg/ml.

***In vitro* anticancer activity PHM-EE**

Effect of PHM-EE on viability of MCF-7 cells

The dose-dependent inhibition of cell viability of breast cancer cell MCF-7 is shown in figure 3. The cells treated with 1000 µg/ml concentration of PHM-EE showed only 6.79% cell viability. The IC_{50} value of PHM-EE against MCF-7 cell viability was found to be 7.8 µg/ml. Representative image of cells in control group and cells treated with 7.8 µg/ml and 1000 µg/ml concentration of PHM-EE is shown in figure 4. In control group, the cells were confluent and showed normal morphology. However, cells treated with 7.8 µg/ml and 1000 µg/ml concentration of PHM-EE showed irregular morphology. Some cells in these groups were in round shape which shows the cell death.

Effect of PHM-EE on DNA fragmentation in MCF-7 cells

DNA was intact in control cells whereas DNA ladder formation was visualized in agarose gel electrophoresis in PHM-EE treated cells (Figure 5). Such DNA ladder formation shows the induction of DNA fragmentation by PHM-EE treatment in MCF-7 cells.

DISCUSSION

The ethanolic extract prepared from polyherbal mixture made up of dry powder of turmeric, tulsi, licorice, black pepper and dry ginger called PHM-EE showed *in vitro* antioxidant activity and anticancer activity against MCF-7 cells. Polyherbal formulations are gaining importance to be studied as potential anticancer agent in recent years due to advantage of synergistic effect that could be imposed by various medicinally-important phytochemicals present in such formulation.[13-23] The polyherbal mixture in the present study was prepared by using turmeric, tulsi, licorice, black pepper and dry ginger, individually all these herbals are well-known for its antioxidant and anticancer properties. The active ingredient, curcumin present in turmeric has inhibited cell proliferation and induced apoptosis in breast cancer cells.[27] Also, curcumin acted as antiangiogenic, anti-invasive, and anti-metastatic agent. In addition, Curcumin was found to reverse the chemotherapeutic resistance in doxorubicin as well as paclitaxel-resistant breast cancer cells.[28-30] Apart from curcumin, other active principles present in turmeric such as turmerone, elemene, furanodiene, cyclocurcumin, calebin A, and germacrone are reported to possess anticancer activity.[31] Tulsi was reported for its anticancer effect against breast cancer through triggering mammalian target of rapamycin (mTOR) pathway.[32] Also, previous study has shown tulsi inhibited matrix metalloproteases thereby hindered the growth and progression of breast cancer.[33] Various phytochemicals with anticancer property were found to be present in tulsi such as luteolin, sinapic acid, xanthomicrol, salvigenin, gallic acid, catechin, chlorogenic acid, caffeic acid, rutin, kaempferol, trans-β-Ocimene and linalool.[34] Plethora of studies have shown the cancer preventive and cancer therapeutic effect of triterpenoid glycyrrhizin present in licorice.[35] Various mechanisms involved in anticancer property of glycyrrhizin are cell cycle arrest, anti-estrogenic effect, induction of apoptosis, immunomodulatory effect, inhibition of metastasis, modulation of various pathways (Nuclear factor-kappa B pathway, MAPK signaling pathway, PKC/ERK pathway, PI3/Akt pathway).[36-38] Also, glycyrrhizin protects against chemotherapy-induced toxicity to other organs.[37] The chalcone compounds, isoliquiritigenin and licochalcone A are other phytoconstituents present in licorice which have anticancer potential and preventive effect of tumor metastasis.[40-42] Piperidine and piperine, the two major compounds present in black pepper with anticancer property.[43,44] Piperine was found to interfere in phosphatidylinositor-3-kinase/Akt signaling pathway thereby inhibit the proliferation of breast cancer cells. Also, piperine inhibit matrix metalloproteases thereby prevent the invasion of breast cancer cells.[45] On the other hand, piperine free black pepper extract which contain alkaloids, phenolic compounds and lignan was also found to have anticancer potential against breast cancer cells through increasing oxidative stress and up-regulation of tumor protein p53.[46,47] Zingerone is a nontoxic important extract of dry ginger which is not detected in fresh ginger. Zingerone have antioxidant anticancer property, strong anti-inflammatory, and antimicrobial properties.[48,49]

Altogether the presence of numerous therapeutically active phytoconstituents in the polyherbal preparation PHM has shown the anticancer effect against MCF-7 cells in the present study. DNA fragmentation is considered as the hall-mark of cells undergoing apoptosis.[50] The treatment of cells with PHM-EE has induced DNA fragmentation in MCF-7 cells which in turn indicate that the cells are undergoing apoptosis. Nevertheless, PHM-EE has shown antioxidant effect through scavenging of radicals such as DPPH and NO in *in vitro*. Such antioxidant effect of PHM-EE would have significant impact such as: i) restriction of tumor progression by resisting mutation; ii) decreasing the concentration of reactive oxygen species which act as signaling molecule in the progression of cancer; and iii) protection of healthy cells against the chemotherapy or radiotherapy-mediated toxicity in healthy cells.[51,52] The results from the present study show that PHM-EE could be used as adjuvant therapy along with chemotherapeutic agents used at present. Previous studies have also shown that, compounds such as vitamin C, vitamin E, retinoic acid, N-acetyl cysteine capsaicin, curcumin, cucurbitacin, gambogic acid, apigenin, chaetocin, chrysin, luteolin, myricetin, quercetin, plumbagin, pentylisothiocyanate, neferine and piperlongumine with antioxidant property could also act as potent anticancer agent.[49, 53-57] Also, other polyherbal formulations have shown both antioxidant and anticancer property.[20, 52, 58] Similar to these studies, in the present study, the PHM-EE has also shown both antioxidant and anticancer activity.

CONCLUSION

Polyherbal mixture made up of dry powder of turmeric, tulsi, licorice, black pepper and dry ginger was extracted using ethanol to obtain PHM-EE. PHM-EE showed antioxidant effect in *in vitro* through scavenging of free radicals such as DPPH and NO. Nevertheless, PHM-EE showed anticancer activity against MCF-7 cells which

was observed as reduction in cell viability and induction of DNA fragmentation. Overall, dual functional roles that is antioxidant as well as anticancer effect was shown by PHM-EE, which shows the wide-spectrum application of polyherbal formulation, PHM in cancer treatment that warrants further *in vivo* studies.

Legend to figures

Figure 1. The *in vitro* antioxidant activity of PHM-EE. Dose-dependent DPPH scavenging activity of PHM-EE (A) and positive control BHT (B) is shown.

Figure 2. The *in vitro* antioxidant activity of PHM-EE. Dose-dependent NO scavenging activity of PHM-EE (A) and positive control ascorbic acid (B) is shown.

Figure 3. The *in vitro* anticancer activity of PHM-EE against breast cancer cells MCF-7. Cell viability after treatment of MCF-7 with different concentrations of PHM-EE for 24 h is shown. The result was expressed as % cell viability.

Figure 4. Image of MCF-7 cells from various groups. Representative phase contrast image of MCF-7 cells in control and cells treated with PHM-EE – 7.8 µg/ml and 1000 µg/ml is shown. The cells in control group are confluent and healthy. The treatment of cells with PHM-EE has induced apoptosis.

Figure 5. DNA fragmentation analysis of MCF-7 cells treated with PHM-EE. Lane M-100 bp DNA maker, Lane T- DNA from 7.8 µg/ml of PHM-EE treated cells, Lane C- DNA from control cells.

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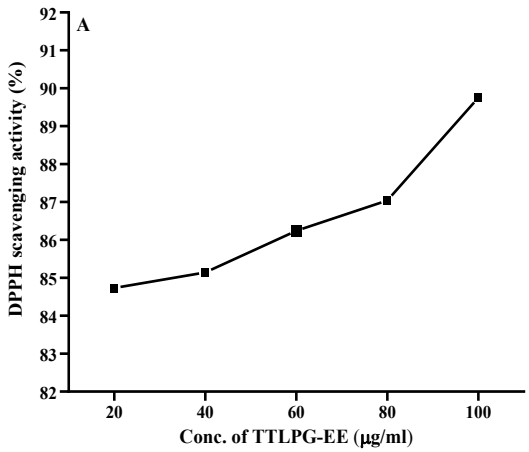
Figure 1



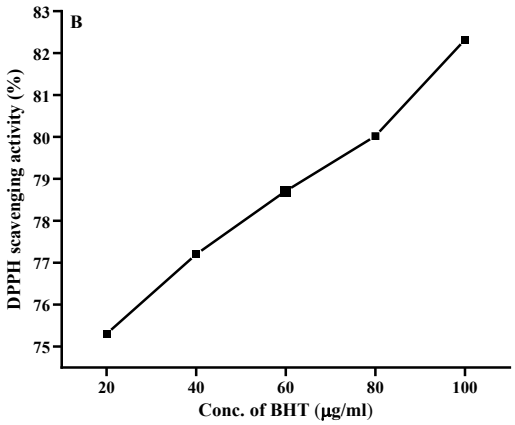


Figure 2

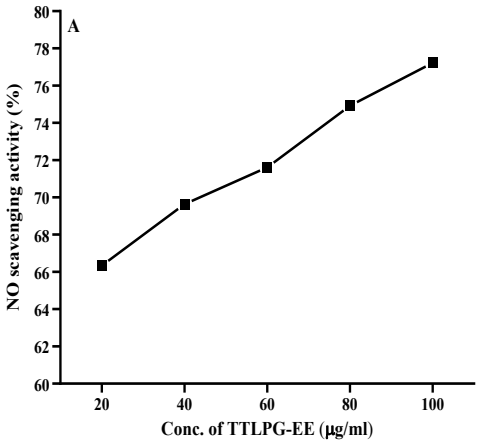


Figure 3

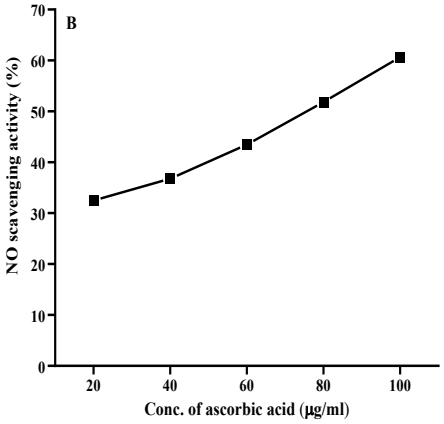


Figure 4

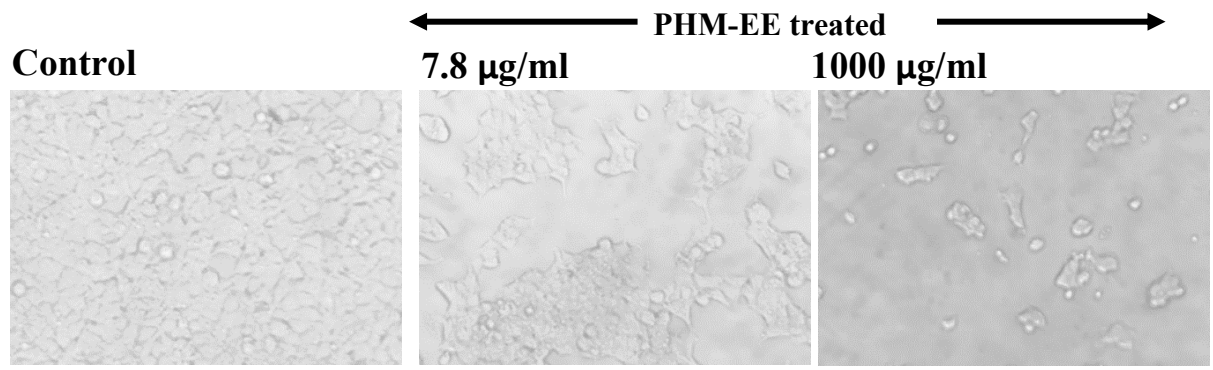


Figure 5