Abstract
Natural products and associated combination therapy have gained prominent role in decreasing the adverse effects of synthetic drugs engaged in the severity of colon cancer. D-limonene a dietary monoterpene and hispolon a bioactive polyphenol proven to be anticancer agents independently against several cancers. The current study is designed to examine complimentary anticancer effect of D-limonene-hispolon concoction in COLO-205 and HCT-116 cell lines. Collectively, our cell viability, cell migration, clonogenic tests and CompuSyn analysis results exemplified that the combination of D-limonene and hispolonnatural products eminently effective against colon cancer cell lines. Gastric cancer patients are reported to develop severe side effects due to the currently available chemotherapy, our combinational anticancer therapy bydietary natural compounds would be highly beneficial to the patients.

Key words: Colorectal cancer, D-limonene, Hispolon.

Introduction
Misregulation of different biological pathways resulted from the genetic mutations, infections, environmental factors, etc., cause various diseases in the body. One of such dreadful disease is cancer, it is one of the major causes of mortality despite immense research in the cancer therapy and prevention worldwide [1]. Among all the cancers reported, colorectal cancer grabs at most attention as this is 2nd leading cancer in both women and men[2]. Majority of colorectal cancers (CRCs) are sporadic, while 10% of them have genetic background. It is demonstrated that the aberrations in Ras and PI3K signalling pathway leads to development of CRC with enhanced risk of tumor[3]. Hence, essentially these multi signalling pathways targeted with multidrug combinations would be highly beneficial. Natural compounds with anticancer activity and pleiotropic properties are involved in better chemo preventive and/or therapeutic alternatives [4].

A monoterpene natural compound D-limonene with a lemon-like odor available in several citrus oils like orange, lemon, lime, mandarin, and grapefruit. The food companies have been using D-limonene additives for flavor and fragrance. D-limonene is also used by clinicians for dissolving cholesterol containing gallstones [5], gastro esophageal reflux disorders, to relieve heartburns, and gastric acid neutralization in the stomach [6]. It is exemplified that D-limonene is involved in regulating many cellular targets in cancer cells such as modulating chemical carcinogenesis, immune modulation, apoptosis, and antioxidant activity. D-limonene is an effective natural compound in preventing the growth of numerous cancer types including lymphomas [7], mammary [8], gastric [9], liver [10], lung [11] and prostate cancer [12] in preclinical cancer models.

Hispolon is a polyphenol isolated from various fungal species such as Phellinus igniarius, Phellinus linteus, and Inonotushispidus[13, 14,
It is identified as an effective natural compound that show antiviral [16], hepatoprotective [17], immunomodulatory [18] and anti-proliferative activities [19, 20, 21] in different models. The role of hispolon is also demonstrated in induction of apoptosis, suppression of metastasis, and cell cycle[22, 23, 24, 25]. In 2008, Wei chen et al., demonstrated the ability of hispolon against gastric cancer and also demonstrated its reduced cytotoxic effect on the normal cells [26].

In the current study, we investigated the role of hispolon, D-limonene and the combination of hispolon and D-limonene against the colon cancer cell lines. Cell viability and cytotoxic assays results demonstrated that the synergistic effect of hispolon and D-limonene is shown significant effect against the COLO-205 and HCT-116.

Materials and Methods

The human colon cancer cell lines HCT 116 and COLO 205 was gifted from Dr. Royal Suresh (IIT, Chennai), RPMI 1640, McCoy’s 5a, Fetal bovine serum (FBS) were purchased from GIBCO Ltd (Life Technologies TM., Grand Island, NY). MTT [3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide], Hispolon, D-Limonene were purchased from Sigma (St. Louis, MO, USA). Stocks were prepared in dimethyl sulfoxide (DMSO) stored at -20°C until use. All other chemicals of analytical grade were purchased from Sigma, USA.

Culturing and maintenance of COLO 205 and HCT 116 cells : COLO 205 and HCT 116 Cells were cultured respectively in RPMI 1640, McCoy’s 5a medium with 10% heat-inactivated Fetal-Bovine Serum and 1% antibiotic solution (10,000 units of Penicillin, 10 mg Streptomycin and 25g amphotericin / ml). Cells were maintained in dimethyl sulfoxide (DMSO) stored at -20°C until use. All other chemicals of analytical grade were purchased from Sigma, USA.

Determination of cell viability : MTT assay is used to determine the effect of hispolon and/or D-limonene on the cell viability of colon cancer cells. Concisely, cells were seeded at a density of 1 X 10^4 cells per well in 96-well plates and treated either with D-limonene or hispolon and also with their combinations, at specified concentrations for 24 and 48 hr. For combination effects the cells were treated with both the drugs concurrently as well as sequentially. In concurrent treatment, first the cells were treated with hispolon(10, 50, 100, 150, 200, 250, 300 μM) and D-limonene (100, 500, 1000, 1500, 2000, 2500, 3000 μM) separately and in case of combinations D-limonene and hispolon (1000+50, 1000+100, 1500+50, 1500+100) were taken for 24 and 48hr continuously. Whereas in sequential treatment, cells were pre-treated with either D-limonene or Hispolon for 12hr, followed by the exposure to the other agent for a total of 24 and 48hr. After that cells were incubated with the MTT reagent (5 mg/ml in DMEM) for 3hr at 37° C, followed by solubilization of the formazan crystals with DMSO for 10min on shaker. Absorbance was measured at 570 nm using a microplate analyzer (iMark Microplate Absorbance Reader, BioRad) [27]. The percent cell viability was calculated using the following

% Cell viability = OD of sample /OD of control X 100

In vitro scratch assay : In vitro scratch assays are particularly appropriate for the cell migration analysis in the cell biology. Cells were seeded at a density of 5x10^4 in 6 well plates and the monolayer of 80% confluent cells were treated for 48hr with hispolon, D-limonene and with their combinations respectively. The concentration of serum in the complete media was decreased to diminish cell proliferation, but used sufficient enough to prevent cell detachment and/or apoptosis. After 48hr of treatment, the medium was removed and a scratch was created with a sterile p200 pipette tip and washed twice with 1X PBS to remove floating cells and plates were incubated after adding complete media with 5% FBS. Cell migration was monitored by capturing images at 0, 24 and 48hr using a bright field microscope (Olympus CX21FS1) [28]. Gap area was measured relative to the total cell-covered area with Wimasis image analysis software. The drug treatment effect was measured by a reduction in the % cell migration at each time interval compared
to untreated control using the % Cell migration formula; % Cell migration = (Scratch area at 0hr- Scratch area at specific time point)/Scratch area at 0h x100.

**Clonogenic assay:** The clonogenic assay is used to determine the ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large colony which is noticeable to the naked eye. Cells were seeded (5×10⁴) in 6-well culture plates and treated with various doses of drugs either single or in combination, as indicated for 48hr. Then COLO 205 and HCT 116 cells were trypsinised and approximately 100 cells were seeded for each drug concentration into new and fresh 6-well culture plates and incubated for another 3 weeks with change of fresh media once in three days. The Colonies were exposed to glutaraldehyde (6% v/v) stained with crystal violet (0.5% w/v) for 30 minutes and counted under a microscope (Olympus CX21FS1) [29].

**Analysis of combination effects:** Further the anticancer effect of Hispolon and D-limonene combination in COLO-205 and HCT-116 cells were analyzed using CompuSyn software [30] for synergism, additive or antagonistic effects based on Combination Index (CI). If the Combination Index (CI) of more than 1 indicate antagonistic, CI is equal to 1 indicate additive and CI is less then 1 indicate synergistic effect.

**Statistical analysis:** The obtained results are presented here as the mean ± standard deviation (SD) from three independent experiments. Differences were evaluated by the one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. The level of significance was set at p < 0.05.

**Results and Discussion**

Natural products are proven as most reliable and effective sources for novel anticancer agents. Prognostic utility of natural compounds in colorectal cancer are currently being investigated [31]. In the current study we demonstrated the synergistic effect of D-limonene and hispolon anticancer activity against COLO-205 and HCT-116 cancer cell lines.

The combinational effect of hispolon and D-limonene was initially screened by its cell viability using MTT assays. Cells were treated with increasing doses of D-limonene and Hispolon (10-300 Î¼M) for 24 & 48hr. Upon drug treatment, cells exhibited significant difference in cell viability compared to control (P ≤ 0.05). In dose and time dependent manner both the drugs tested individually and they inhibited the cell proliferation (Fig. 1). Under these experimental conditions, the calculated IC50 values in COLO-205 were at 1042 Î¼M and 100 Î¼M for D-limonene and hispolon respectively; whereas in HCT-116 the IC 50 values for D-limonene and hispolon were at 1850 Î¼M and 190 Î¼M respectively. Both COLO 205 and HCT-116 were treated with different combinations of the drugs sequentially as well as simultaneously added and cell viability was identified as described before by MTT assay (27). The combination of varying doses of D-limonene and hispolon produced maximum antiproliferative activity at 48hr when compared with the treatment of either agents alone in both the cell lines. The combination of D-limonene and hispolon (LIM+HIS) at a concentration of 1500+100 produced highest antiproliferative activity in COLO-205; whereas in HCT-116 also the combination at a concentration of 1500+100 showed highest activity (Fig. 2.A.D). Antiproliferative effect was more when the cells were exposed to both drugs simultaneously than sequential treatment (84% vs. 70% or 65% for COLO-205: Fig. 2.A.B.C) and (86% vs. 68% or 62% for HCT-116: Fig. 2.D.E.F).

**Effect of D-limonene and hispolon on cell migration:** The cell migration experiments were conducted to study the synergistic effect of D-limonene and hispolon in COLO-205 & HCT-116 cell lines (Fig. 3.A.B). Lower doses of D-limonene (250, 500 Î¼M) and hispolon (25, 50 Î¼M) and their possible combinations were chosen for the study to minimize the cytotoxicity. In control group cell migration into the scratch was 100% in 48hr resulting complete closure of the gap, whereas
Fig. 1. Effect of D-limonene and hispolon on cell viability showing the effect of D-limonene and hispolon on cell viability in COLO-205 (A,B); HCT-116 (C,D) CRC cell lines. Cells were treated for 24 & 48 hr and viability was determined by MTT. Data were expressed as mean ± SD (n=3). *p < 0.05.

Fig. 2. Effect of D-limonene and hispolon combination on cell viability. Combination effect of D-limonene (LIM) and hispolon (HIS) on anti-proliferative activity in COLO-205 & HCT-116 cells. A.D: Cells were treated with D-limonene in combination with hispolon at the same time. B.E: Cells were pre-treated with D-limonene for 12hr followed by exposure to hispolon. C.F: Cells were pre-treated with hispolon for 12hr followed by exposure to D-limonene. Cells were treated for a total of 24 & 48hr and viability was determined by MTT. Data were expressed as mean ± SD (n=3). *p ≤ 0.05.
drug treatment alone or in combination caused a significant inhibition of cell migration in both the cell lines ($P<0.05$). The maximum inhibition of % cell migration observed at 48hr was 63.65, 59.45 in COLO-205 and HCT-116 cells respectively at the highest concentration of LIM+HIS (500+50 μM).

**Combination of D-limonene and hispolon**

**Fig. 3.** D-limonene and hispolon inhibits cell migration. Effect of D-limonene and hispolon combinations on cell migration in COLO-205 (A); HCT-116 (B) cell lines. Cells were treated for 48hr and scratch images were captured at 0, 24 and 48hr and analyzed as described in materials and methods. The percent cell migration was expressed as mean ± SD (n=3). $^*p < 0.05$

**Fig. 4.** D-limonene and hispolon inhibits colony formation. Effect of D-limonene and hispolon combinations on colony formation of COLO-205 and HCT-116 cell lines. A: The percent inhibition of colony formation at different drug combinations expressed relative to untreated cell control considering as zero; B: Images of colonies that were stained with 0.5% crystal violet reagent. The data were expressed as mean ± SD (n=3). $^*p \leq 0.05$.
inhibits colony formation: Clonogenic assay was performed to study the effects of D-limonene (250, 500 μM), hispolon (25, 50 μM) and their combinations (Fig. 4.A). Represent images of the assay were shown in (Fig. 4.B). Both the drugs at high concentrations resulted a significant inhibition of colony formation (%) compared to control (P<0.05). Drug combinations were more effective than either of the drugs alone. LIM+HIS at high concentration (500+50) showed maximum inhibition of 53.66; 51.76 in COLO-205 & HCT-116 cells respectively.

Assessment of synergistic anticancer activity of D-limonene and hispolon: CompuSyn software (32) Drug combinations was analyzed by CompuSyn software and CI values were generated to determine synergy, additive or antagonistic effects. LIM+HIS on cell viability was strongly synergistic (CI<1) at 1000+50; 1000+100 in COLO-205 when the drugs were exposed at the same time. In HCT-116 also at 1500+50; 1500+100 produced strong synergism in the same format. In case of pretreatment formats all the combinations were shown to be antagonistic (CI >1) in both the cell lines (Table 1.A). In COLO-205 the combination shows strong synergism on inhibition of cell migration at 250+50; 500+25 and 500+50; whereas it shows a weak synergistic interaction on colony formation at the dose of 500+25. However, in HCT-116 the combination at 500+25; 500+50 produced strong synergism on

Table 1. Combination Index (CI) values of D-limonene and hispolon combination effect on COLO-205 and HCT-116 cell lines A: Effect of combination treatment on anti-proliferative activity. Cells were treated with the drug combination simultaneously as well as sequentially as described in material & methods. B: Effect on cell migration and colony formation. Synergistic interaction is determined if CI<1, an additive interaction if CI=1 and antagonistic if CI >1.

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inhibition of cell migration and clonogenic ability (Table 1). Together our results demonstrated that the combination treatments of hispolon and D-limonene was more significant than the independent drug treatment. It is also evident from our results that exposure of both drugs at the same time was more effective than pre-treatment format. Results confirmed that COLO-205 cells are more sensitive to anticancer activity of the hispolon and D-limonene.

**Conclusion**

In conclusion, overall our results revealed that the combination treatment of hispolon and D-limonene elicits synergistic anticancer effect in colon cancer cell lines (COLO-205 and HCT-116). These kind of combined natural compound treatments may have further clinical utility for treating colon cancer, however the current findings require additional experimental evidence to identify anticancer effects of hispolon and D-limonene combinations in different cancer cell lines. Here we suggest that present study may be valuable to identify potential anticancer compounds and this kind of efficacious natural drugs dietary products would help to combat against dreadful human diseases without side effects.

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**Conflict of Interest**

The authors declare no conflict of interest.

**References**


Anticancer potential of D-limonene and hispolon against colon cancer cell lines


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