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Article

Cytotoxic Efficacy of Indigo and Yellow 2G with Vitamin C on the HepG2 Cell Line

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Abstract: We aimed to examine the effect of indigo and yellow 2G (Y2G) dyes, that are also commonly used as food additive and colorant, individually and combination with vitamin C on HepG2 human liver carcinoma cell line proliferation in vitro. MTT assay was applied to investigate the effect of dyes individually combined with vitamin C on HepG2 cells by treatment with different concentrations of these dyes for varying exposure times and performed. Regarding the exposure of HepG2 cells to indigo and Y2G dyes for 24, 48 and 72 h, 50 and 500 µg/ml (p=0.001 and p=0.003 respectively) of indigo at 24 h and 500 µg/ml (p=0.012) at 72 h; it showed that 5 µg/ml of Y2G for 24 h (p=0.004) and 1 µg/ml for 48 and 72 h (p=0.000, p=0.001) had the highest cytotoxic effect. Also, the results indicated that, especially the combinations of "indigo + vitamin C" significantly increased cytotoxic effects on the HepG2 cell line (p=0.003). The obtained findings provide an exciting insight into the cytotoxic effects of indigo on HepG2 cells, and how they might work together to combat HCC's aggression.

Keywords: Hepatocellular cancer; MTT; Indigo; Yellow 2G; HepG2.

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1. Introduction

Indigo or Indigotin ($C_{16}H_{10}N_2O_2$) is water-insoluble (2 ppm) vat dye that contains anthraquinone (including polycyclic quinones) and indigoids (Gupta and Suhas, 2009). Indigo, with a Lethal Concentration 50 (LC_{50}) of Leuciscus idus (Golden orfe) > 10.000 mg/l-96 h is one of the oldest known blue dyes and produced naturally or synthetically in large quantities (20 million kg per year) by plants. Currently indigo has an extensive potential for colouring yarn in the manufacture of blue jeans and denim products (Bankole et al., 2017; Yalcuk and Okcu, 2017). Since the time when Chinese scholars had discovered that this dye can reduce fever and detoxify the blood, there have been some studies on the pharmacological properties of the indigo dye began (Qi-Yue et al., 2020). Recent studies have been reporting that indigo has anti-inflammatory, antioxidant and antibacterial properties as well as activities such as regulation of immune response (Gaitanis et al., 2018; Naganuma, 2019). Further, it has been indicated that indigo and its derivatives (such as indirubin, isatin and triptantrin) are used in hemoptysis, epistaxis, chest pain, aphtha, infantile convulsion and cancer treatment, which have an inhibitory effect in various types of cancer (e.g. leukemia, skin and prostate) treatment with inhibitory effects (Zhu et al., 2019; Sun et al., 2021).

Like indigo dye, anionic, synthetic monoazo yellow 2G (Y2G), also known as Acid Yellow 17, is another widely used textile dye, food additive and colourant that is a potential threat to textile workers or those other handlers (Okçu et al., 2019). Y2G is easily dissolved in water and resistant to biodegradation and microbial cell absorption due to the presence of sulfonate functional groups (Kannaujiya et al., 2021). It is also known that exposure to Y2G can damage the organisms' respiratory system (shortness of breath, dermatitis, etc.), cardiovascular and nervous system (Ortiz-Monsalve et al., 2017). In addition, Y2G has mutagenic and tumorigenic effects on bacteria and yeast, affecting genetic material in the somatic cells of mammals, causing problems during reproduction and growth (Ashraf et al., 2013). It is intriguing to see how Y2G, whose beneficial or detrimental effects are not entirely obvious from the literature, may impact the cancer cell when used alone or in combination with vitamin C at various doses and durations. Although we are unsure of the nature of this effect, we believe that our study will help to answer this question in some way. Although it has similar usage areas to indigo dye, to our knowledge, there appears no studies on the effects of Y2G on the treatment of diseases such as cancer

in publicly available literature. It is obviously clear that studies with such focus are necessary due to the direct or indirect exposure of people to Y2G being used in various fields.

While some of the materials used in the industry are known to cause cancer, the relationship between occupational risk and cancer is often unclear. Workers in the textile, construction, and chemical industries exposed to dyes and solvents are reported to be particularly at high risk for liver cancer (Rapisarda et al., 2016; Singh and Chadha, 2016). For instance, hepatocellular carcinoma (HCC) is one of the leading forms of cancer worldwide and remains a global health concern. It is estimated that by 2025, more than 1 million people will be affected by liver cancer every year (Gilles et al., 2022). It is the fifth most common form of cancer in adult males, while the ninth most common form of cancer in adult females (Gilles et al., 2022; Tunissiolli et al., 2017). The need for a limited treatment option in the HCC has arisen due to the high incidence of carcinoma and post-surgical reproduction rates and the ineffectiveness of chemotherapy and radiotherapy in advanced HCCs. Current drugs used in cancer treatment usually have serious side effects. Therefore, researchers have been investigating the effectiveness of naturally occurring bacterial or fungal toxins or plant-based agents in cancer treatment as alternative or complimentary to synthetic drugs (Huang et al., 2020).

Several studies have shown that different plant derived components, natural products, and/or agents increase the death of cancer cells in vitro and in vivo environments (Talib et al., 2020; Iqbal et al., 2017). Plant extracts form the basis for multiple prescriptions in traditional alternative medicine and appear to be a complementary approach to treating cancers. In addition, some studies have interestingly shown that these positive effects increase with the use of vitamin supplements (Yuan et al., 2016; Samtiya et al., 2021).

Ascorbic acid (Vitamin C) is a natural product that has been shown to improve the antineoplastic activity of some chemotherapeutic drugs and influences the cell proliferation of antioxidant properties (Hunyady, 2022). Vitamin C, an enzyme cofactor found in plants and some animals, is a soluble antioxidant in water. Additionally, new research has demonstrated that vitamin C enhances the cytotoxic effect of components used against cancer (Hunyady, 2022; Luo et al., 2022).

Considering all these, with this study we aimed to determine the cytotoxic effects of indigo and Y2G alone and in combination with vitamin C on human liver carcinoma cell (HepG2) line proliferation. The reason for choosing the HepG2 cell line in this study is that it is compatible with cell morphology that models human liver cancer in vitro. To determine whether indigo and Y2G dyes have an effect on HepG2 cells, we incubated this cell line with different concentrations of these dyes for varying exposure times and performed viability using the MTT test method. We believe that, examining for the first time the effects of different doses of indigo and Y2G dyes on HepG2 cells with the combination of vitamin C, the result from our study contributes significantly to the field by offering novel data.

2. Materials and Methods

We confirm that the cell cultures used in the study do not require ethics committee approval. The test procedure used in this study is schematically depicted in Figure 1.

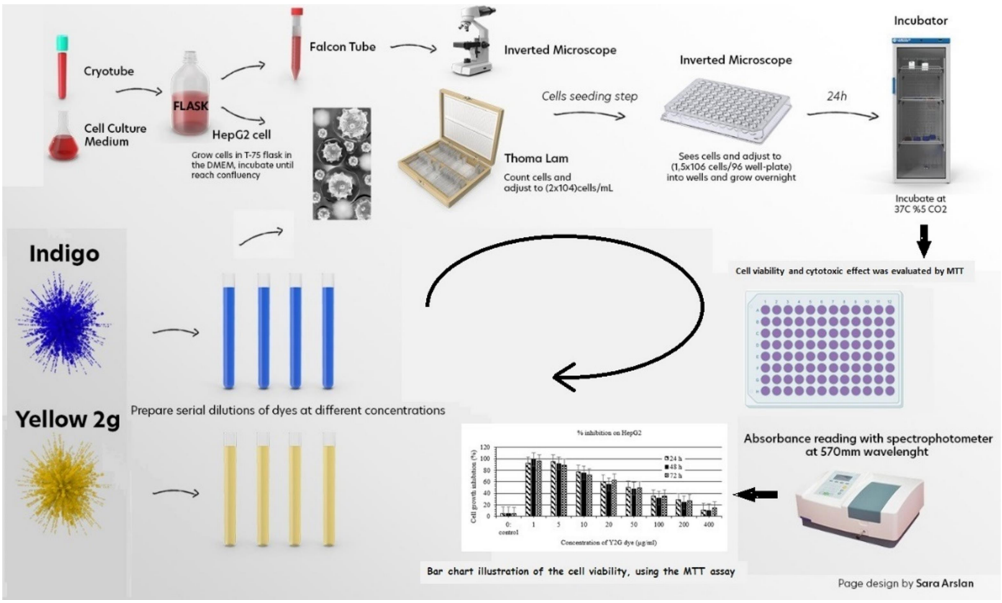


Figure 1. The summarization of the experimental procedure.

2.1. Chemicals and Reagents

Synthetic indigo and Y2G dyes obtained from two different suppliers: Sigma-Aldrich, (Darmstadt, Germany) and a local textile company (Realkom Düzce, Turkey). The Y2G and indigo from Realkom were, respectively, 100% and 40% water soluble. The dyes were used in the tests as received from the suppliers without further purification of which chemical structure and properties are given in Figure 2.

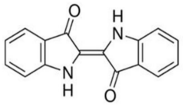
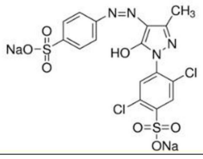
Name	Molecular structure	Chemical structure	Molecular weight (MW, g/mole)	λmax (nm)	Cas No
Indigo or Indigotin		$C_{16}H_{10}N_2O_2$	262.26	613	482-89-3
Yellow 2G (Y2G) or Acid Yelow 17		$C_{16}H_{10}Cl_2N_4Na_2O_7S_2$	551	390	6359-98-4

Figure 2. The chemical structure and properties of synthetic dyes (Okcu et al., 2019).

Other chemicals and reagents used in the study were as follows: fetal bovine serum (FBS) (Gibco, Life Technologies, South America), Dulbecco's Modified Eagle Medium (DMEM) and ascorbic acid or Vitamin C and trypan blue (Sigma-Aldrich, Darmstadt, Germany), trypsin-EDTA and penicillin-streptomycin (Kibbutz Beit-Haemek, Israel), dimethyl sulfoxide (DMSO) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl- tetrazolium bromide (MTT) (Serva, Germany).

2.2. Cell culture conditions

Human liver carcinoma HepG2 (ATCC HB-8065) cells were obtained from the ATCC (American Type Culture Collection, Manassas, VA, USA) in cryotubes packed with dry ice and transferred and preserved in liquid nitrogen (N_2) until the time of their use. First, these frozen cells were thawed in water bath (Wise Bath, Wisd Laboratory Instruments, Korea) at 37ºC until it completely liquified. Then, the cells were cultured in T25 flasks (Nunc™ EasYFlask™ Cell Culture Flasks, Thermo Fisher Scientific, USA) each containing 10 ml previously prepared medium composed of 9 ml DMEM, 1000 U penicillin/streptomycin, and 1ml FBS. Then, the cells in flasks were cultured in a CO_2 incubator at 37°C for 24 h under a humidified atmosphere of 5% CO_2 + 95% air supplied from a dedicated gas cylinder. The medium was replaced with the fresh one after 24 h. Before the cells being subjected to the dye treatment, they were passaged according to HepG2 supplier company (ATCC HB-8065) recommendations and continued to be cultured until they reached 80-90% confluency determined by an inverted microscope (Zeiss AX10, UK) as shown in Figure 3.

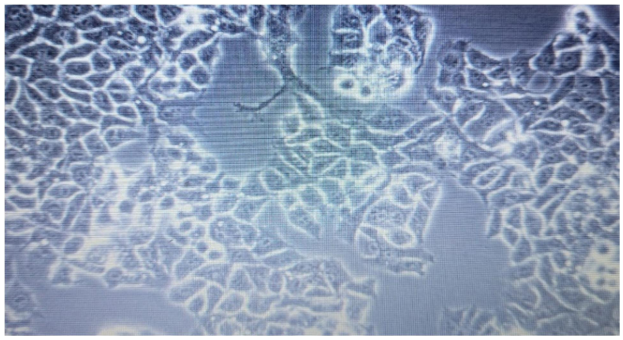


Figure 3. HepG2 cell density

Trypan blue was used to establish cell viability and cell quantification according to the method described in the Reference (Strober, 2015). Thoma cell counting chamber (Thoma, Iso Lab, Germany) was used to calculate cell viability and cell numbers. Then, 100 µL of cultured medium was transferred into 96-well plates already containing 100 µL DMEM to yield nearly 1.2 x105 cells/well culturing density. These 96-well plates were incubated in the same incubator used for initial culturing for 24 h to ensure good cell attachment.

2.3. Treatment of HepG2 cells by indigo and Y2G dyes and a combination of vitamin C

Indigo and Y2G dyes from different suppliers (Sigma and Realkom) were applied to inoculate 96-well plates in the section above in doubles alone and together with Vitamin C with the concentration and the volumes (total sums of 256 samples=2 dyes x 2 suppliers x 2 repeat x 4 Vitamin C x 8 concentration) as shown in Table 1. The inoculated 96-well plates were kept at 37 ºC in a 5% CO_2 with saturating 95% humidity in cell culture incubators to be used later in the cytotoxic effect tests described below.

Table 1. The concentration and volumes of reagents applied to HepG2 cells

Concentration (µg/ml)		1	5	10	20	50	100	200	400	500	1000
100 ml each of	Indigo	√	√	√	√	√	√			√	√
	Y2G	√	√	√	√	√	√	√	√		
	Vitamin C							√			

2.4. Determination of cytotoxic effect using of MTT assay

The 96-well plates as described in the section immediately above were subjected to MTT assay (Mossmann, 1983) after 24, 48 and 72 h incubation. Cells incubated in 10% FBS were used as positive control and the viability of the cells was determined. 10 µl of MTT reagent was added to every well of each 96-well plate and incubated at 37 ºC in a 5% CO_2 with saturating 95% humidity for 4 hours. Then 100 µl DMSO was added to each well for solubilising formazan precipitates. Absorbance (A) values/optical density were read at 570 nm (Mossmann, 1983) with a spectrophotometer (colorimetric reader, Multiskan GO 51119300, Thermo Fisher Scientific, USA) and a growth curve was plotted. The percentage of A at 570 nm of the experimental group to that of control group was used as cell viability criteria as given in Equation 1.

$$\text{Cell viability (\%)} = \frac{\text{OD of the experimental group at 570 nm}}{\text{OD of the control group at 570 nm}} \times 100$$

The average of the absorbance values for the control wells was considered as 100% viable cells. The optical density (OD) values of all the wells were used to calculate the proliferation effect (in percentage) of the treatment compounds (i.e., indigo, Y2G, and vitamin C) to the cell's viability.

2.5. Statistical analysis

As mentioned above, the tests were carried out in duplicate (in total of 256 tests). The Kolmogorov-Smirnov (K-S) Test was used to determine whether the datasets' normal distribution was present. Number of units (n), percentage (%), mean standard deviation (x SD), and median (Q1-Q3) data were used to represent descriptive statistics. Statistical analysis was performed using Student's t-test when comparing only two groups of data and one-way analysis of variance when comparing more than two groups' data using computer package program (SPSS 22.0, Statistical Package for the Social Sciences, Chicago, IL, USA). $p<0.05$ was considered as statistically significant for the cytotoxicity proliferation effect. The results were analysed by using GraphPad Prism 5.0 software (GraphPad Software, San Diego, USA).

3. Results and Discussion

The results obtained are shown in Figure 4 and Figure 5. As it can be observed from the results indigo and Y2G decreased cell viability in HepG2 cells in a dose-and time-dependent manner. However, it was determined that cell viability decreased only at effective certain doses (ie. for indigo 50 and 500 µg/ml, for Y2G 1 and 5 µg/ml). The Sigma indigo and Y2G dyes at the same doses have no statistically significant proliferation effect (data not shown) and have lower impact on the rate of proliferation than Realkom ones (p=0.421). The data show that both dyes are effective on tumour cell number, and that the cytotoxic effect is more dominant in indigo than in Y2G ($p<0.01$). Sigma dyes have different effects depending on both time and dose. The cell proliferation of HepG2 is greatly inhibited by 50 µg/ml and 500 µg/ml dose of indigo for 24 h (p=0.001 and p=0.003, respectively), however 5 µg/ml dose of Y2G for 24 h (p=0.004) (Figure 4a and Figure 4b).

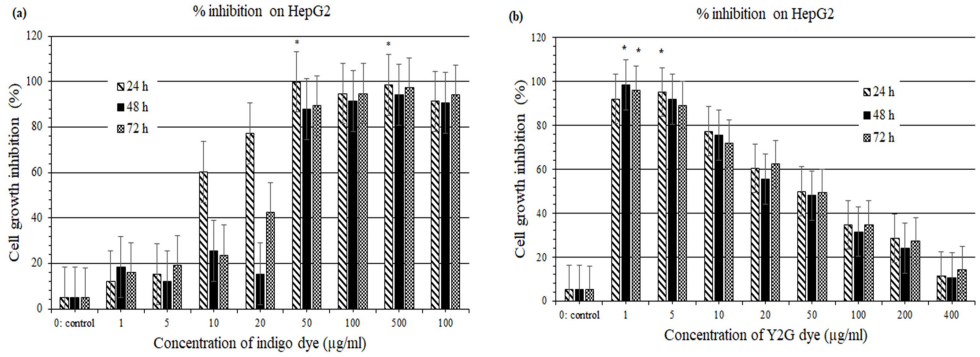


Figure 4. The anti-growth effect using MTT assay after treatment with the indigo (a) and Y2G (b) dye for 24, 48 and 72 h.

With respect to other time periods, indigo at 50 µg/ml dose shows insignificant effect at 48 and 72 hours, except for 500 µg/ml dose for which it is effective at 72 hours (p=0.012). It is apparent from the data that Y2G is cytotoxically more effective (>%90) compared to indigo and that its effective dose is 5 µg/ml for 24 hours but 1 µg/ml for 48 and 72 hours (p=0.000, p=0.001, respectively).

As shown in Figure 4a and 4b, treatment of the HepG2 cell line with both Indigo and Y2G displays concentration and time dependence time dependent decline in cell viability. The particularly suppressive effect begins in the first 24 hours and continues throughout the application of further extended times ($p<0.01$ vs. the control group). In particular, the high dose of indigo has a significantly suppressive effect (p=0.002 versus control group). Y2G show effectiveness in lower doses (1 and 5 µg/ml). It is apparent from the results that presence of vitamin C with both dyes had a positive effect on HepG2 proliferation. Figure 5a show that the effect of vitamin C on indigo's effect is significant (p=0.001). In the case of the Y2G and vitamin C combination, there appears some effect on the cell proliferation, but it is not that significant (p=0.981; Figure 5b).

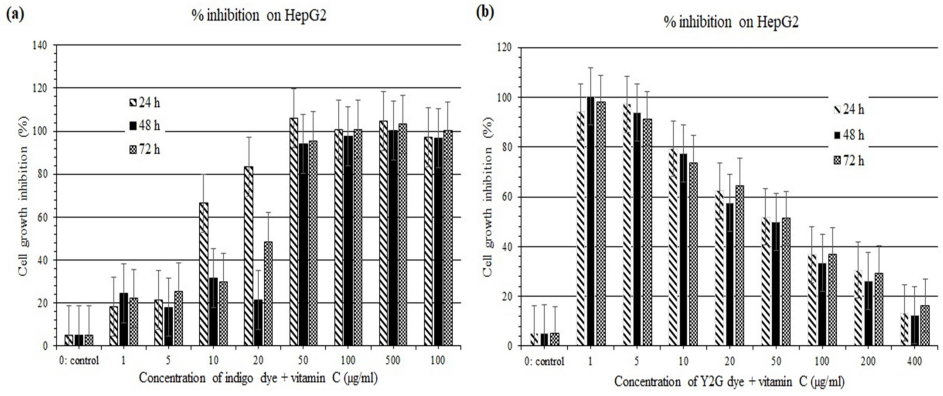


Figure 5. The anti-growth effect using MTT assay after treatment with the indigo + vitamin C (a) and Y2G + vitamin C (b) for 24, 48 and 72 h.

The MTT test results (Figure 4a and Figure 5a) also confirm that indigo effectively reduces cell proliferation, both with increases in concentration and time. Also, the combination of indigo with vitamin C showed a statistically significant effect on HepG2 cells at three different time intervals (24, 48 and 72 h) ($p=0.003$; Figure 5a).

In the present work we investigated the effects of two dyes (indigo and Y2G) on the cytotoxicity in HepG2 cell lines. Although the positive effects of indigo on cancer treatment are known, no data on liver cancer cell line at different doses and times to our knowledge is available in the literature. The results presented herein showed that, for 24 h, while the indigo dye could inhibit the growth of HepG2 at higher doses (50-500 µg/ml) in agreement with findings of Shimizu et al. who reports that the effects of indigo are accelerated at concentrations higher than 1 µg/mL, Y2G dye at lower doses (1-5 µg/ml). Additionally, their study states that treatment of cells for 24 h with high concentrations of indigo (2.62-26.2 µg/ml) inhibited cell numbers (Shimizu et al., 2021). It is believed that the reason of the dose-dependence is due to the pharmacological effects of indigo such as anti-inflammatory, anti-tumor and anti-oxidant properties (Qi-Yue et al., 2020). Furthermore, Knapp et al. report significant inhibitory effects at the higher concentrations such as 1000, 500 and 100 µg/mL by comparing the effects of indigo extract on MCF-7 and MCF-12A breast cancer cells for 48 hours of exposure (Knapp et al., 2018). Therefore, our results confirm the evidence of inhibition at higher doses of indigo.

Although it has been mentioned that Y2G, which has similar properties to indigo dye, has certain effects on cancer (Qi-Yue et al., 2020), the information on this subject is quite limited. Our results presented that the lower doses of Y2G are more effective on HepG2 cell line than those of the high doses of indigo dye. We believe that this is due to the reason that Y2G is more toxic as result of its chemical structure. It should be considered that while Y2G has a toxic effect on cancer cells at low concentrations, if administered at high dosages, it may have cancer-promoting effects. This is assuming that Y2G is toxic in the literature, however this issue is unclear. Another possibility is that Y2G, which was ineffective at low levels, failed to exert any effect at high dosages because of the nonlinearity of the dose-response curve. This second strategy seems highly improbable given that Y2G exhibits negative health impacts. However, we recommend more research to be done on this subject to verify us believe further. Exposure time is also crucial factor for the cytotoxicity effect of dyes on HepG2 cells. While, in the first 24 hours, Indigo showed cytotoxic effects at doses of 50-500 µg/ml, for 72 hours it demonstrated effectiveness at only 500 µg/ml. According to these results, the effective doses of indigo vary depending on the exposure time. For both in vitro and in vivo studies, we concluded that attention should be paid to dose as well as time of exposure. Similarly, it is apparent from the data we presented in the current study, Y2G is cytotoxically more effective compared to indigo. Additionally, its effective dose is 5 µg/ml for 24 hours but 1 µg/ml for 48 and 72 h as shown in Figure 4a and 4b.

Finally, cell viability was decreased depending on the dosage and exposure time of indigo and Y2G dyes in the treatment of the HepG2 cell line. The inhibition percentage started within the first 24 hours and continued throughout the 48th and 72nd hours as long as the application continued as given in Figure 4a and 4b.

Similar to many traditional alternative medicine prescriptions of plant extracts and other natural compounds are now being seen as complementary to current clinical cancer treatments, applications of vitamin supplements have also been receiving increasing attention (Iqbal et al., 2017; Talib et al., 2020). For cancer treatment, vitamin C has occasionally been utilized as an unconventional supplement (Wang et al., 2016). Lee et al. explains that high doses (1.25 to 20 mM) of vitamin C combined with common anti-cancer drugs might have therapeutic benefits against breast cancer cells (Lee et al., 2019). Similarly, Danisman-Kalindemirtas reports that the ability of drugs in fighting cancer was enhanced by vitamin C and that the vitamin C supplements decreased cell viability extremely (Kalındemirtaş, 2022). It is quite clear from the result presented here that presence of vitamin C with both indigo and Y2G dyes had a positive effect on HepG2 proliferation. Although, the effect of Y2G and vitamin C combination is statistically not that significant, there appears some observable impact on the cytotoxic effect due to this combination.

4. Conclusion

To our knowledge, this is the first study where indigo and Y2G dyes alone and in their combination with vitamin C have been evaluated for their cytotoxicity in HepG2 cells for different time periods (24, 28, and 78 hours) and doses (as shown in Table 1) with the aim of examining their potential role on cancer. The results demonstrated that the cytotoxic effect of indigo and Y2G dyes alone or in combination with vitamin C, especially for indigo, on the human liver cancer cell (HepG2) is dependent on the dose of these compounds and exposure time of the cells. Although, the change in the cytotoxic effect of each dye within its group was statistically insignificant with regards to their source (sigma or local textile company), indigo was found comparatively better. The results presented herein, thus, further support indigo as a potential

drug candidate against human liver cancer cells in vitro. The Y2G effect has not been so far studied widely on the cell line viability. This suggests a need for further studies. Indigo and Y2G show ability inhibiting cell viability in human hepatocyte cancer cells/hepatic cancer cell lines, especially the combinations of "indigo+vitamin C". Thus, we recommend further future studies to determine the pathway and how these components can act as anti-proliferative effects in HepG2 cells.

Conflict of Interest

The authors have no conflict of interest to declare.

Financial Disclosure

Authors declare no financial support.

Authors' Contributions

Conceptualization, S.K.; data curation, S.K.; formal analysis, S.K.; funding acquisition, S.K., G.D, and T.P.; investigation, S.K., and G.D.; methodology, S.K., and G.D.; resources, S.K. G.D, and T.P.; supervision, S.K.; writing-original draft, S.K., and G.D.; writing-review and editing, T.P. All authors have read and agreed to the published version of the manuscript.

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