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Research Article

Increased Anti-Proliferation Performance of NanoChitosan-Moringa Seeds Extract and Co-Treatment with Doxorubicin in Liver Cancer Cells

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ABSTRACT

Hepatocellular carcinoma (HCC) with high epidemiological report data. Pathogenesis in HCC also involves several signaling pathways. This study aims to evaluate the in vitro activity of Moringa seed NanoChitosan against Hep G2 liver cancer cells and Co-Treatment with Doxorubicin. Initially, nanoparticles were prepared by extracting Moringa seeds, formulating them into nano chitosan, and then characterizing the compounds and particle sizes. The IC_{50} dose was investigated using the MTT assay. Then, the IC_{50} dose was confirmed in more detail through immunofluorescence, betatrophin gene, several genes in the Wnt-βcatenin-CyclinD1 proliferation pathway, and the addition of the apoptotic effector Caspase-3 using RT-qPCR analysis. Each treatment used a single dose of NCH-Mosee and co-treatment or combination with 4 µg/ml doxorubicin. The IC_{50} dose was 994 µg/ml in single treatment and 649 µg/ml in combined treatment with Dox. Hep G2 showed a decrease in the expression level of each parameter measured with increasing single dose and combination treatment ($p < 0.050$). Histologically, cells shrank, betatrophin expression was inhibited, and luminescence was seen, which decreased with increasing dose. In conclusion, NCH-Mosee with dose-tracking toxicity combined with Dox can suppress the viability of Hep G2 cells.

1. Introduction

Globally, the latest epidemiological data reports that liver cancer or hepatocellular carcinoma (HCC) is the second cancer among the ten cancers causing death in the world (Globocan-WHO 2020). In general, the etiological factors of HCC include chronic hepatitis C and B, alcoholic and non-alcoholic fatty liver, as well as metabolic syndrome (Jindal *et al*. 2019). The pathogenesis of HCC involves hyperactivation of several signal pathways, such as MEK/ERK (Moon & Ro 2021), EGF/EGFR (Huang *et al*. 2014), mTOR (Ferrín *et al*. 2020), HGF/c-Met (Wang *et al*. 2020), Wnt/β-catenin (Monzavi *et al*. 2019).

One candidate biomarker used to detect HCC is betatrophin (lipasin/C19ofr80/ANGPTL8), a liver-derived hormone that plays a significant role in lipid metabolism and glucose metabolism disorders (Chen *et al*. 2015). Another research also found antiproliferative and apoptotic effects of betatrophin on pancreatic and ovarian cancer cells (Taherkhani

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et al. 2020; Sahmani *et al*. 2022). Betatrophin also contributed to the Bcl2 activation pathway that could inhibit apoptosis (Navaeian *et al*. 2021). This aligns with the research results by (Tseng *et al*. 2014; Susanto *et al*. 2019). Stating that betatrophin was highly expressed both in vitro and in vivo. Other research also indicated that betatrophin functioned as a regulator of HCC, primarily through the Wnt/β-catenin pathway; the presence of betatrophin could suppress WIF-1 (Wnt Inhibitory Factor -1) (Monzavi *et al*. 2019). Meanwhile, WIF-1 acts as an inhibitor of the Wnt/β-Catenin signaling pathway, triggering proliferation (Luo *et al*. 2018).

The standard clinical treatment for cancer, especially HCC, mainly uses doxorubicin as the first-line drug therapy (Cox & Weinman 2016; Dubbelboer *et al*. 2019). However, doxorubicin brings about side effects, including hypertension, as well as being toxic to other organs, such as kidney and heart problems, thyroid disorders, itching, redness, and alopecia (Thorn *et al*. 2011; Ajaykumar 2021). Therefore, to minimize the use of these drug doses, a drug made from natural ingredients or herbal medicine is needed as a combined therapy for cancer, especially HCC (Huang *et al*. 2014).

In this research, the herbal candidate used was *Moringa oleifera* (MO) seeds. MO is a miracle tree and tropical plant that is becoming a development target to overcome stunting. However, the use of Moringa seeds as a candidate for natural anti-cancer ingredients or natural products, which are a rich source of lead compounds for drug discovery, including anti-cancer drugs, is rarely minimally reported (Vasanth *et al*. 2014; Ezhilarasi *et al*. 2016). Moringa acts as a potential inhibitor of cancer cell proliferation. Interestingly, MO is a plant that is easy to find (cosmopolite). Previous preliminary research showed that Moringa leaf powder had great potential as an anti-inflammatory and fibrosis green material in in-vivo studies based on the component analysis results (Susanto *et al*. 2018). Moreover, they screened phytochemically using GC-MS to determine active compounds (volatile-non-polar), with the results of obtaining four dominant groups of compounds in Moringa seeds, namely hydrocarbons, esters, fatty acids, and alcohols (Susanto *et al*. 2023). The dominant compounds were in the high acid group, which was 66%, especially oleic and gallic acids, which were 63.53%. This is similar to another research that states that moringa seeds contain high levels of fatty acids, including 60% oleic acid (Cretella *et al*. 2020; *Leone et al*. 2016). Furthermore, the bioactive compound content of Moringa seeds identified through LC-MS was found to be several non-volatile-polar compounds, such as flavonoids, glycolic, and olefins (Premi & Sharma 2017).

The implementation of MO as a therapeutic drug for cancer is expected to have a good effect in inhibiting the development of cancer. Therefore, the authors used a drug delivery system in the form of chitosan nanoparticles. Chitosan is a polyelectrolyte drug delivery system that can increase drug solubility with controlled distribution (Pateiro *et al*. 2021; Wani *et al*. 2022). Besides that, chitosan is also known to have the ability to act as an adsorbent, so it can be used to absorb harmful substances in several unwanted components (Utami *et al*. 2022). Chitosan nanoencapsulation coating can be conducted using several techniques, including ionic gelation. The working principle of this technique relies on the electrostatic attraction of polyelectrolytes between molecules having opposite charges to form a hydrogel (Nurlaela *et al*. 2020). In encapsulation, the coating material (chitosan) coats the core material, namely the active substance (Wani *et al*. 2022). Nanoencapsulation using chitosan can show acceptable stability in the gastrointestinal environment and has the potential as a carrier for orally administered drugs, and the material used is also non-toxic (Mohammed *et al*. 2017; Rad *et al*. 2021). The urgency of the research is the potential of Moringa seeds as an anti-cancer herbal plant. A pretty good bioavailability is required to maximize Moringa's bioactive compounds for bioprospecting to medical use as herb medicine. Therefore, the encapsulation process by chitosan is expected to protect and increase the biological effects of the active compounds of Moringa seeds resulting from *n*-hexane extraction in hindering proliferation levels and betatrophin expression in liver cancer cells.

2. Materials and Methods

2.1. Plant Collection, Extraction, and Nanoparticle Synthesis

Moringa seeds were obtained from PT. Universal Moringa Plantations, Malang City, East Java. Then, the chitosan nanoparticles were extracted and formulated at the Pharmaceutical Preparation Formulation Laboratory, Universitas Muhammadiyah Malang. The chitosan nanoparticles were made using the ionic gelation method (Hoang *et al*. 2022). Moringa seed *n*-hexane extract was mixed with acetic acid and chitosan solution. The

surfactant used was sodium tripolyphosphate, which had been filtered and sonicated to reduce the size of the membrane particles. The first characterization was particle size using the PSA (particle size analyzer) test at the Integrated Laboratory and Research Center, University of Indonesia. The following characterization was the compound content at the Pharmacy Laboratory of Universitas Brawijaya using the GC-MS method.

2.2. Cell Culture and Treatment

This in vitro study was conducted at the Parasitology Laboratory, Faculty of Medicine, Universitas Brawijaya. Hep G2 cell line or human hepatocellular carcinoma cells were purchased from ATCC Catalog Number HB-8065. Those cells were cultured using a complete medium containing Eagle Minimum Essential Medium (EMEM) (ATCC-No.30-2003) and 10% fetal bovine serum (Gibco No 16000044). This research has been approved by The Animal Care and Use Committee, Universitas Brawijaya, Indonesia, with the certificate of ethics number (048-KEP-UB-2023).

2.3. MTT Assay

In the MTT test, researchers carried out two stages: IC_{50} dose screening and proliferation testing. First, to screen the IC_{50} dose, five doses of chitosan *n*-hexane extract from *Moringa oleifera* seeds (NCH-Mosee) nanoparticles were used, including 125, 250, 500, 1,000, and 2,000 µg/ml. This research also targeted the combined therapy of NCH-Mosee with the drug doxorubicin/dox, having a known IC_{50} dose of 8 µg/ml on Hep G2 cells. Therefore, to minimize the use of dox, the dose for combined therapy toxicity was 4 µg/mL added to each of the five doses of NCH-Mosee (15.75, 31.25, 62.5, 125, and 250 µg/ml). The Hep G2 cells were cultured until 80% confluent in 96-well plates and then incubated for 48 hours. Those cells were then analyzed using an MTT reagent (Biovision, Catalog No. 2809-1G) and read at a wavelength of 570 nm using an Elisa Reader. Second, in the proliferation test, one type of dose was used, both in the combined treatment of 600 µg/ml + dox 4 µg/ml and the single NCH-Mosee treatment of 900 µg/ml. The researchers used different treatment incubation times, including 24, 48, and 72 hours. Then, the data were read at a wavelength of 570 nm.

2.4. Measurement of Betatrophin Expression and Proliferation Signaling via Wnt

Various treatment doses from combined therapy (H3 dose: 600 µg/ml + dox 3 µg/ml) or single therapy (300 (H1), 450 (H2), & 600 (H3) µg/ml) were injected into Hep G2 cells that had been 80% confluent on a 24-well plate + coverslip for imaging with an incubation time of 24 hours. Then, screening was carried out using betatrophin target parameters measured using immunofluorescence and RT-qPCR. Immunofluorescence single staining employed ANGPTL8/Betatrophin Rabbit anti-Human Polyclonal Antibody (Lsbio, Catalog No. LS-C201171-0.1), mouse monoclonal secondary antibody IgG1 Rhodamine (Santa Cruz Biotech, Catalog No. sc-57606), and DAPI (Roche, No. 10236276001). Subsequent measurements used RT-PCR to target proliferation signaling via Wnt/ βcatenin. Materials used in the RT-PCR method included QIAzol Lysis Reagent (Qiagen, ID: 79306), Revertra-Ace TOYOBO cDNA synthesis kit (Toyobo Japan, Id: FSQ-301), and Sensyfast SYBR No-Rox Kit (Meridian Bioscience, BIO-98005). Previously, Hep G2 cells were grown on 6-well plates and waited until confluent before being given combined and single therapy treatment. The parameters measured in the RT-PCR method are listed in Table 1.

2.5. Statistical Analysis

The data were reported as mean and standard deviation (SD). The statistical significance of data differences between treatments was analyzed using one-way ANOVA ($p<0.05$) and continued by the Post Hoc Tukey Test.

3. Results

3.1. Nanoparticle Characterization

3.1.1. Particle Size Analyzer

The exploration of the synthesis of n-hexane chitosan nanoparticles extracted from *Moringa oleifera* seeds (NCH-Mosee) used the ionic gelation method. This research was also supported by the results of a formulation containing nanoparticle-sized NCH-Mosee (106.8 nm), as shown in Table 2.

3.1.2. GC-MS Analysis

The compounds listed in Tables 3 and 4 were coated with sodium tripolyphosphate, allowing ion exchange between the coating and the bioactive compounds.

3.2. MTT Assay

Subsequently, the exploration of the IC_{50} dose through the MTT test was undertaken, and its results are displayed in Figures 1 and 2, showing that cell

Table 1. List of sequences of target genes (primers)

Gene	Forward (5'-3')	Reverse (3'-5')	Source
B-actin	GCC TCC TGC ACC ACC AAC TG	CCA TCA CGC CAC AGT TTC CC	NCBI: locus NM_001357943 [32]
Wnt	ACC ACA TGC AGT ACA TCG GAG	GAG GTG TTA TCC ACA GTG CTG	NCBI: locus XM_054347758 [33]
Betatrophin	GAG ACT CAG ATG GAG GAG GA	ATG CTG CTG TGC CAC CAT CT	NCBI: locus NM_018687 (Lee *et al*. 2016)
β-catenin	GCC GGC TAT TGT AGA AGC TG	ACT AGT CGT GGA ATG GCA CC	NCBI: locus XM_054345317.1 [35]
CyclinD1	CGT GGC CTC TAA GAT GAA GGA	CCT CGG GCC GGA TAG AGT AG	NCBI: locus NM_053056 [36]
Caspase-3	GCA AAG AAA TCA TTA TCC CCA G	TTT GCT TAT TAC ACA TCC CCA T	[37]

Table 2. Particle size analyzer using horiba zetasizer machine

Calculation results				
Peak no	S.P.Area ratio	Mean	S.D	Mode
1	1.00	472.8 nm	139.6 nm	427.5 nm
2	-	- nm	- nm	- nm
3	-	- nm	- nm	- nm
Total	1.00	472.8 nm	139.6 nm	427.5 nm
Cumulant operations				
Z-average			106.8 nm	
PI			0.617	

Table 3. Content of steroids and triterpenoids in Nanochitosan *n*-hexane extract of *Moringa oleifera* seeds

Compounds/category	Content [µg/g±SD]
Sterols:	
Campesterol	39.05±2.26
Sitosterol	550.87±8.90
Stigmasterol	25.62±1.35
Sum of sterols	615.54
Steroid ketones:	
Sitostenone	25.73±1.50
Stigmasta-3,6-dione	47.21±1.82
Tremulone	20.17±1.40
sum of steroid ketones	93.17
sum of steroids	708.65
Neutral triterpenoids:	
α-amyrin	297.03±8.07
β-amyrin	133.15±5.32
sum of neutral triterpenoids	430.18
Triterpenoid acids:	
maslinic acid	32.11±2.10
maslinic acid methyl ester	n.d.
oleanolic acid	40.76 ±1.17
3-oxooleanolic acid	25.64±1.21
ursolic acid	88.53±2.56
sum of acids	187.04
sum of triterpenoids	617.22
Total	**1325.87**

Table 4. Content of flavonoids and phenolics in Nanochitosan *n*-hexane extract of *Moringa oleifera* seeds

Compounds/category	Content [µg/g±SD]
Flavonols:	
Quercetin-acetyl-glycoside	1.05±1.06
Quercetin-malonyl-glucoside	2.76 ±1.25
Quercetin	5.35 ±1.52
Kaempferol-3-O-rutinoside	7.87±2.90
Kaempferol acetyl glycoside	10.12±1.15
Isoquercetin	n.d.
Astragalin	1.33±1.05
Sum of flavonols	28.48
Flavones:	
Vitexin	2.73±0.80
Rutin	0.93±0.12
sum of flavones	3.66
sum of flavonoids	32.14
Phenolic:	
Naphthoquinones	7.03±1.62
Quinic acid	1.15±0.75
Caffeic acid	0.87±0.14
Chlorogenic acid	n.d.
Gallic acid	n.d.
Coumaroylquinic acid	n.d.
Salicylic acid	n.d.
Luteolin	3.15±1.68
sum of phenolic	12.2
Total	**44.34**

viability experienced death or decreased significantly (p-value<0.05). The calculated graph in Figure 1 shows that the IC_{50} dose is 994 µg/ml.

Furthermore, exploration of the IC_{50} dose through the MTT test was carried out with results as in Figures 1 and 2 showing that cell viability experienced death or decreased significantly (p-value<0.05) by the Anova One Way Test. Based on the calculated graph in Figure 1, the IC_{50} dose is 994 µg/ml.

The generic cancer drug, namely doxorubicin, was minimized based on an IC_{50} dose of 8 µg/ml. Then, it is used in half and given alternately with various doses of NCH-Mosee (Figures 3 and 4). Based on calculations using the line equation formula, the IC_{50} dose was obtained at 649 µg/ml. Furthermore, the proliferation test was conducted using a dose of 600 µg/mL NCH-Mosee combined with 4 µg/ml dox and a single dose of 900 µg/ml NCH-Mosee. The duration of both combined and single treatments was 24, 48, and 72 hours. The results of the proliferation test indicated that as time increased, the ability of NCH-Mosee was

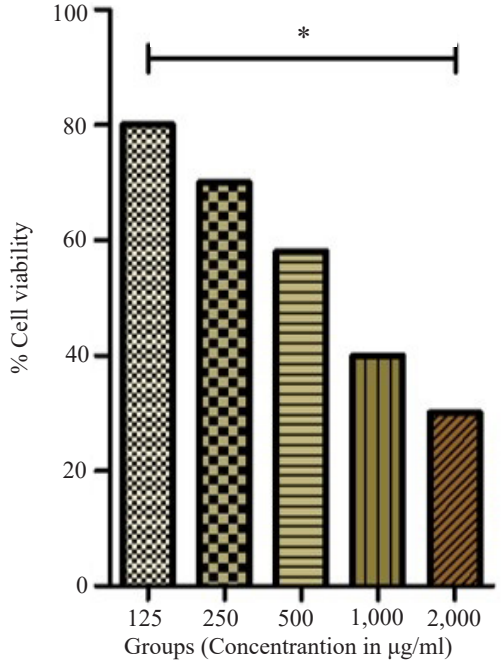

Figure 1. Toxicity test of single treatment (NCH-Mosee). Significantly (p value < 0.05) by anova one way test

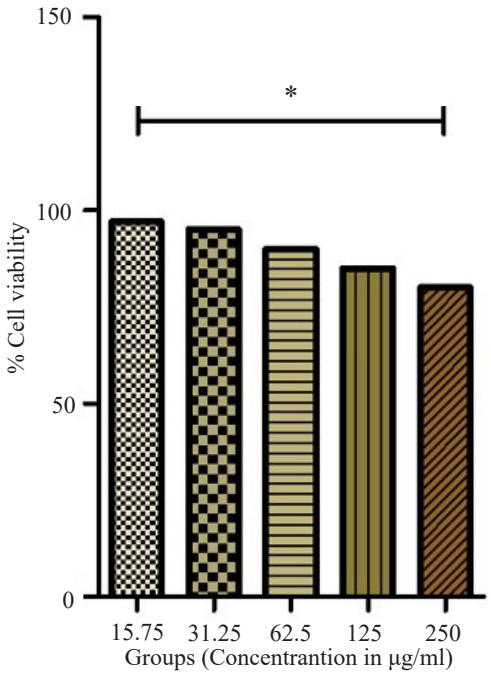

Figure 3. Toxicity test of combination treatment (NCH-Mosee + Doxorubicin). Significantly (p value < 0.05) by anova one way test

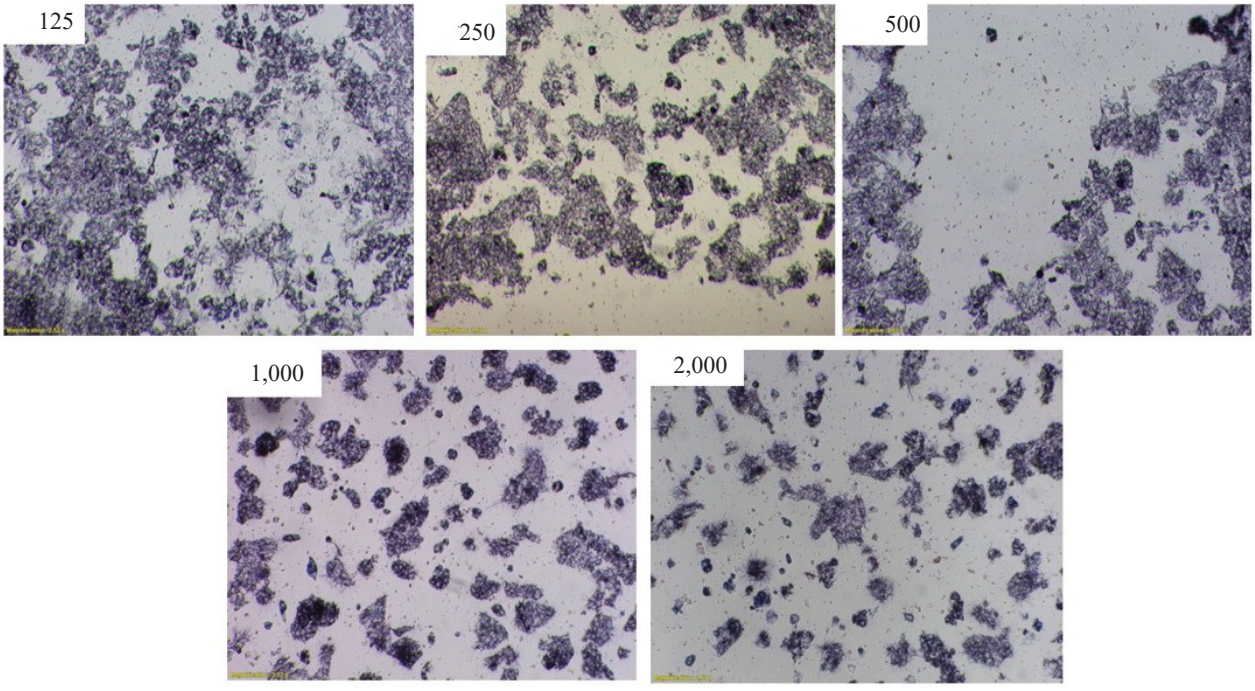

Figure 2. Morphology of toxicity tests of single treatment. Observation under an inverted microscope with 400x magnification

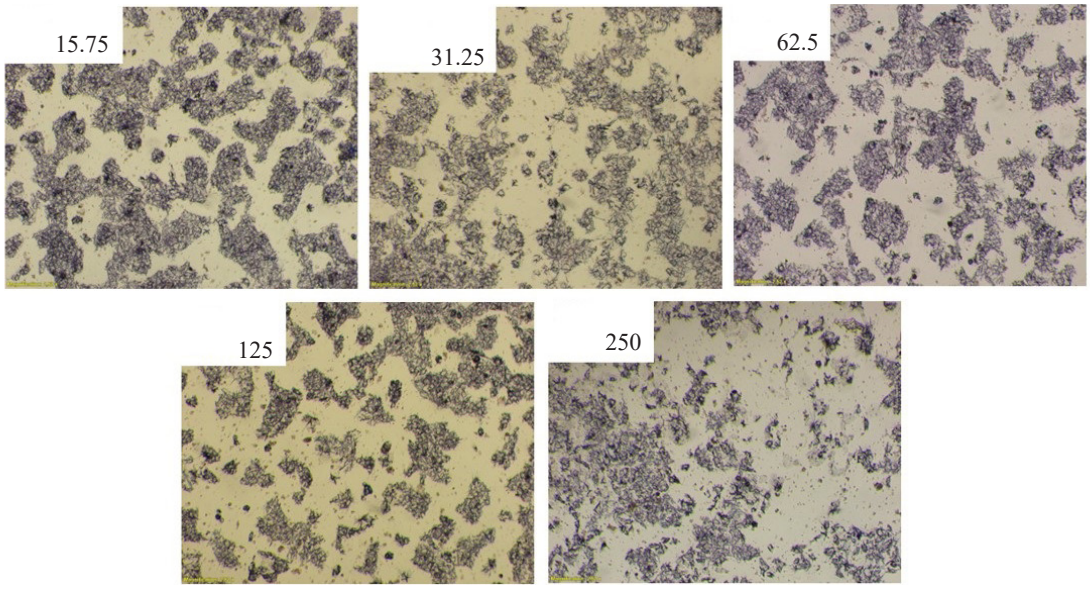

Figure 4. Morphology of toxicity tests of combination treatment (NCH-Mosee + Doxorubicin). Observation under an inverted microscope with 400x magnification

enhanced, and the cancer cell viability was suppressed (Figures 5 and 6).

3.3. Betatrophin Expression and Proliferation Signaling via Wnt

The parameters were used to examine the alleged role of NCH-Mosee in the Hep G2 cell line in the proliferation pathway, cell toxicity, and reconfirmation at both protein and RNA levels in more detail. Especially more details related to Betatrophin expression (Figure 7) to see how it is expressed in this case. Figures 8 and 9 show different signaling paths. The immunofluorescence analysis treatment revealed the relative expression of the Wnt, β-catenin, and CyclinD1 genes contributing to the proliferation pathway was successfully suppressed by varying doses of NCH-Mosee. In contrast, Figure 9 indicates that the expression of the main effector of apoptosis, Caspase-3, is highly expressed in the positive control and significantly increases with the increasing doses of NCH-Mosee used.

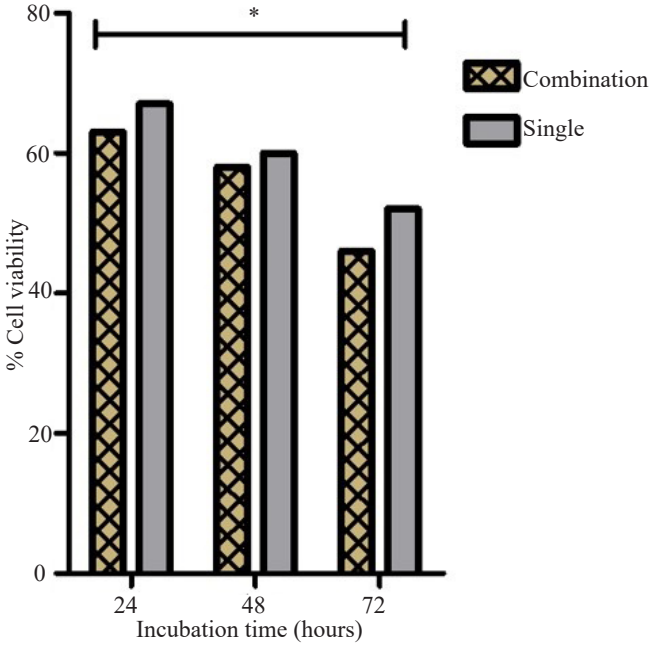

Figure 5. Proliferation assay. Significantly (p value < 0.05) by anova one way test

4. Discussion

The potential of the anti-cancer properties of the extraction using the solvent *n*-hexane has been investigated, and several studies have shown promising results. A study found that the *n*-hexane extract of a plant showed cytotoxicity on breast cancer stem cells through the induction of apoptosis (Pathiranage *et al*. 2021). The extract also can ward off beneficial free radicals since they can contribute to cancer development. Based on the screening results via GCMS (Table 3 and 4), the polar compounds were still found, although they were relatively few. This aligns with the extraction using the *n*-hexane solvent capable of filtering non-polar compounds, such as sterols and triterpenoids (Awotedu *et al*. 2020). *n*-hexane is a non-

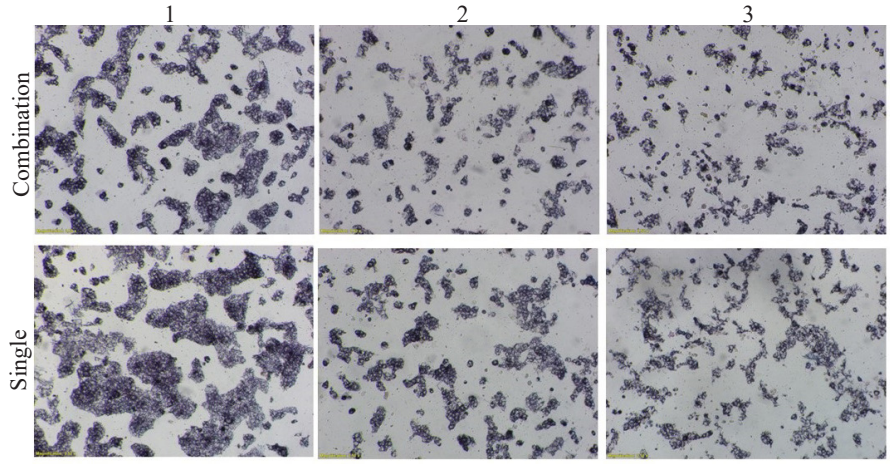

Figure 6. Morphology of proliferation tests (single and combination). Observation under an inverted microscope with 400x magnification

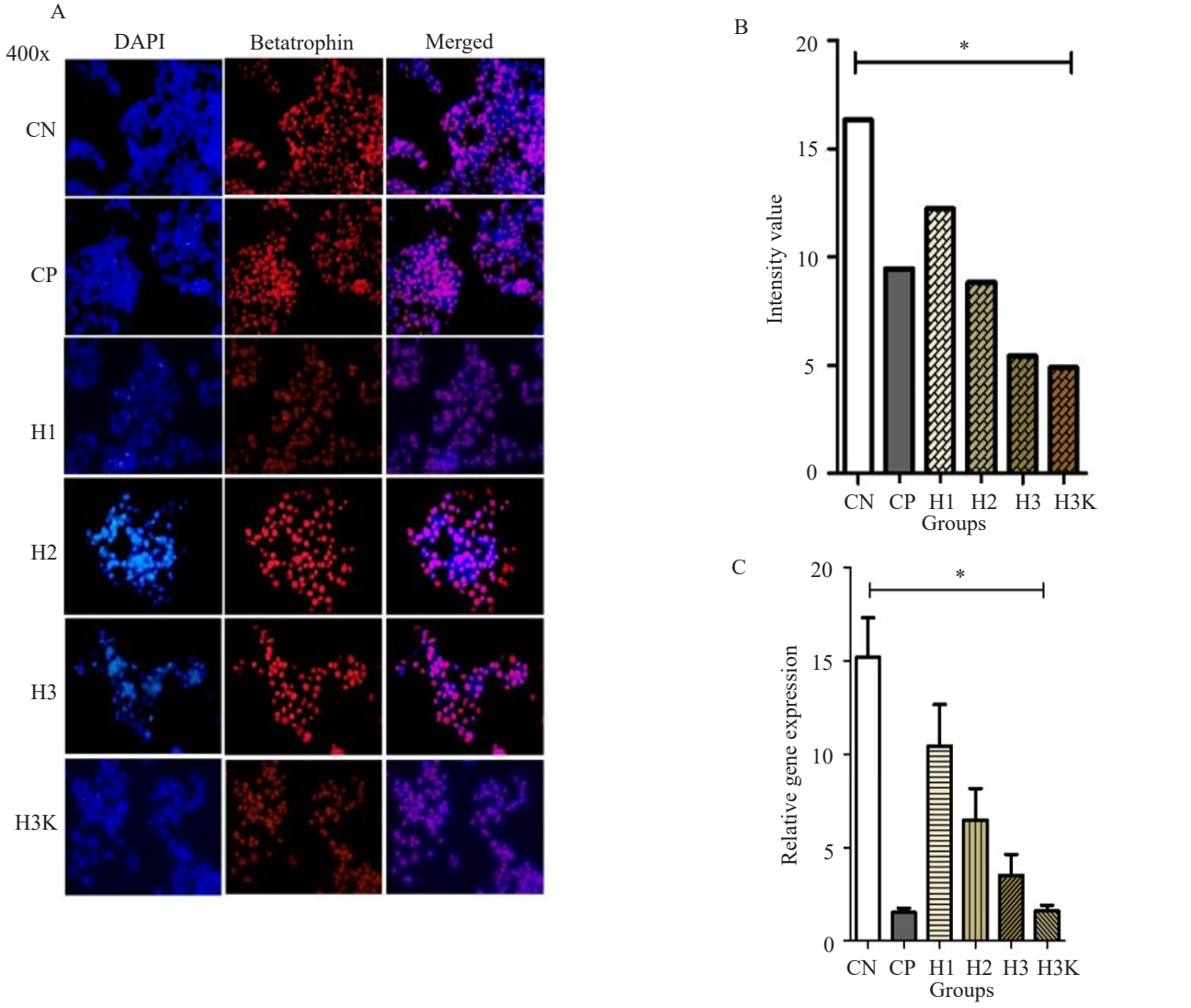

Figure 7. A) & B) Immunofluorescence data and c) RT-qPCR of Betatrophin expression in combined and single treatments. Significance between treatments compared to control ($p < 0.050$) by anova one way test

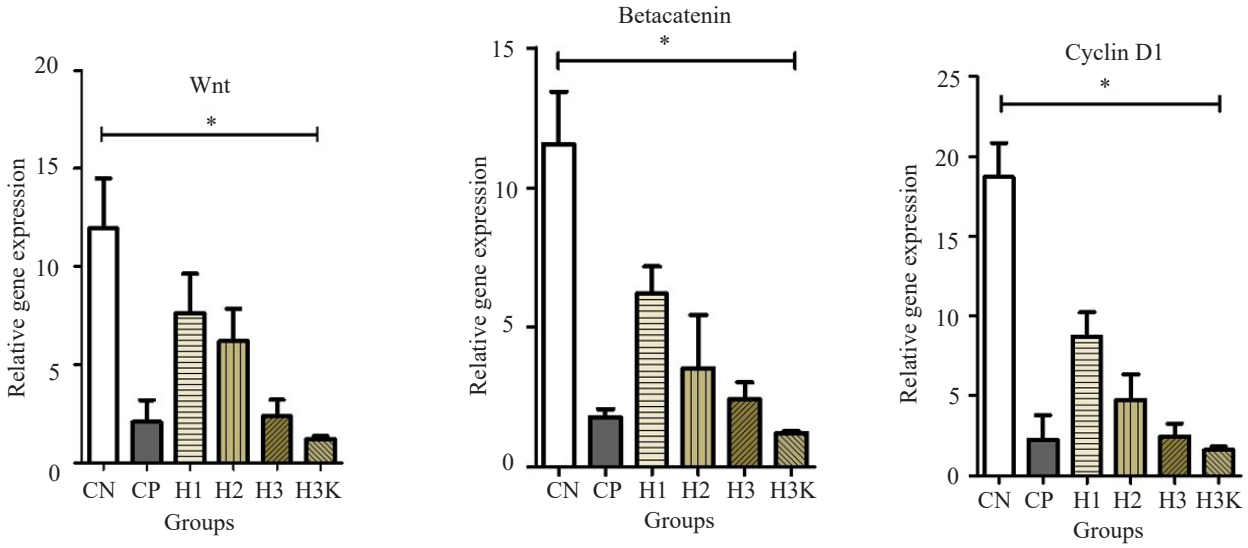

Figure 8. Value of relative gene expression through proliferation process via Wnt/β-catenin pathway. Significance between treatments compared to control ($p < 0.050$) by anova one way test

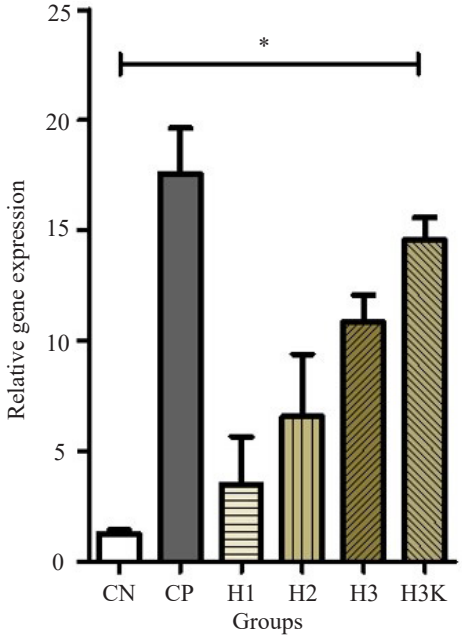

Figure 9. Value of relative gene expression Caspase-3. Significance between treatments compared to control ($p < 0.050$) by anova one way test

polar solvent; thus, *n*-hexane is suitable for extracting non-polar compounds from Moringa plant seeds. However, the extraction of phytochemical compounds from plants showed that non-polar solvents, including *n*-hexane, can extract flavonoids, alkaloids, saponins, and known polar compounds (Bourgou *et al*. 2021; Cravotto *et al*. 2022).

The NCH-Mosee showed that the cytotoxicity levels in Hep G2 cancer cells were upgraded compared to those in the non-treated group. This extract caused morphological changes in apoptosis characteristics and DNA fragmentation in cancer cells, as demonstrated in Figure 8. Likewise, in the positive control and combined treatments through inhibition of topoisomerase II and the performance of the NCH-Mosee target, the caspase activity (a marker of apoptosis in Figure 9) increased, and the cell viability decreased. More DNA damage accumulates, the spheroid will lose its membrane integrity (Flörkemeier *et al*. 2022). This is supported by the immunofluorescence measurements of Betatrophin, in which its role in cancer cases, especially the proliferation pathway, has rarely been proven.

However, Taherkhani *et al*. (2020) Stated that the role of Betatrophin in pancreatic cancer cells is activating the WIF-1 pathway so that the proliferation signaling pathway via Wnt can be inhibited (Taherkhani *et al*. 2020). In contrast, Figure 7 indicates that the level of betatrophin expression decreases as the NCH-Mosee dose increases. Therefore, the role of betatrophin in this proliferation signaling pathway should be investigated more, considering the lack of literacy regarding this matter and betatrophin's dual role in cancer cases, especially liver cancer.

Furthermore, NCH-Mosee activates the main effector in the apoptosis process, namely caspase-3, as seen in Figure 10, as the results of RT-qPCR analysis of Caspase-3. This indicates that apoptosis depends on the induction of NCH-Mosee treatment. This RNA-based molecular exploration confirms the results of the toxicity and proliferation test (MTT Assay) Figure 6. Hep G2 cells experienced up to 40% death at a dose of 1000 µg/ml and optimum treatment at an incubation period of two days or 48 hours to achieve an IC_{50} dose. Meanwhile, Caspase-3 plays a significant role in activating enzymes in the protease pathway, inducing apoptosis and associated with cell death (Silva *et al*. 2022).

The molecules on the Wnt, β-catenin, and CyclinD1 pathways were investigated, considering that they contributed to liver cancer proliferation through various mechanisms (Li *et al*. 2014; Nishikawa *et al*. 2018). In Figure 8, significantly ($p < 0.050$), the three target genes-expression was inhibited as the concentration of the single dose of NCH and the combination (co-treatment) increased. This is in line with other studies showing that inhibition of the Wnt/β-catenin pathway has potential as a target for HCC treatment (Zhang & Wang 2020; Bakrania *et al*. 2023). The inhibition of the Wnt/β-catenin signaling pathway suppresses the proliferation and activation of hepatic stellate cells (HSCs) involved in liver fibrosis and cancer development (Liu *et al*. 2022; Bakrania *et al*. 2023). Both positive control (synthetic inhibitor) and NCH-Mosee (nanoparticle-based natural extract) treatments have shown potential in inhibiting the Wnt pathway, thereby suppressing liver cancer cell proliferation. Both work together to restrain the rate of cancer cell proliferation.

The combined therapies were carried out in this study to minimize the use of generic drugs. In this case, the positive control is Doxorubicin. According to Al-Shafie *et al*. (2023), the IC_{50} dose of doxorubicin/dox in Hep G2 cells is 8 µg/ml (Al-Shafie *et al*. 2023). The dose was reduced by half to 4 µg/ml and then combined with NCH-Mosee. Doxorubicin is a potent chemotherapy drug utilized in the treatment of various types of cancer, including liver cancer (Radu *et al*. 2022). However, its use has several drawbacks and potential side effects. Poor selectivity and adverse reactions for patients are some of the main disadvantages of dox, such as multidrug resistance, hepatotoxicity, and side effects (Ahmed *et al*. 2022; Xu *et al*. 2022; Yildirim *et al*. 2022). Although dox successfully kills the tumor cells, it can harm healthy cells, causing severe adverse reactions.

However, despite the weaknesses of dox, it remains the primary drug in the treatment of liver cancer due to its anticancer solid effect by directly inhibiting topoisomerase II (Taymaz-Nikerel *et al*. 2018; Mutlu *et al*. 2020; Paskeh *et al*. 2021). Therefore, this research aims at reducing the weaknesses of dox, such as developing a nanoparticle-based drug delivery system, namely NCH-Mosee, enhancing selectivity, bioavailability, and reducing side effects, or combination therapies doxorubicin with other drugs to overcome multidrug resistance (Sesarman *et al*. 2021) In this research, Combined therapies with a dox dose of 4 µg/ml + 600 µg/ml could suppress the proliferation rate of Hep G2 cells. However, we need to convey that this study has limitations, including the unavailability of normal cell lines as a comparison (e.g., the NIH 3T3 fibroblast cell line), and the characterization of nanoparticles has not yet tested the efficiency of encapsulation and morphology (TEM and SEM).

In conclusion, NCH-Mosee with dose-tracking toxicity combined with Dox could suppress the viability of Hep G2 cells. This effectiveness is supported by the high content of NCH, such as sterols, steroids, triterpenoids (non-polar compounds), and small amounts of phenolics and flavonoids. Then, the NCH-Mosee with dose tracking toxicity and combined with Dox was applied to measure other parameters, and the results showed potential anti-cancer and anti-proliferation properties by inhibiting the expression of the betatrophin, Wnt, β-catenin, Cyclin D1, as well as triggering apoptosis (Caspase-3) pathways mentioned and proven in the data above. However, the effectiveness of these extracts may vary depending on the type of cancer cell line, and more research is needed to understand those extracts' potential therapeutic applications.

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