Anticancer effect of Triphala extract on the hepatocellular carcinoma cells in mice

[Efecto anticancerígeno del extracto de Triphala sobre las células de carcinoma hepatocelular en ratones]

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Abstract

Context: Triphala is a traditional herbal formulation consisting of dried fruits from three plants namely, Terminalia bellirica, Terminalia chebula and Phyllanthus emblica. Its traditional used to treat many ailments, including various types of cancers, health promotion, and longevity.

Aims: To evaluate the ability of an aqueous extract Triphala (TPL) to inhibit the growth of Hepatocellular carcinoma cells (HepG2) and in mice.

Methods: The anticancer activity of TPL and its composite extracts was assessed in vitro by MTT assay with HepG2 cells. The mice were inoculated with HepG2 cells and then divided randomly into six groups (6 mice/group): control group, positive control group (intraperitoneal with 3 mg/kg body weight doxorubicin), and TPL treatment groups (oral administrated with 50, 100, and 200 mg/kg body weight TPL, respectively). Anticancer activity based on body weight, tumor growth volumes, survival time, relative organ weight, and hematological parameters was determined after administrating the TPL for 14 consecutive days.

Results: The results demonstrated that the TPL extract exhibited antiproliferative activity against HepG2 cells in a time-dependent manner of treatment. The selectivity index (SI) showed that TPL is highly selective (SI>3) against the HepG2 cell line and has no activity against non-tumor cells. Oral administration with TPL extract at the dose of 200 mg/kg body weight inhibited tumor growth volume and increase the survival time. No abnormality of hematological parameters, relative organ weight, body weight, and morphology of organs were observed.

Conclusions: The TPL extract at the test doses was a non-toxic drug. Our finding reveals the anticancer efficacy of TPL extract.

Keywords: anticancer activity; hepatocellular carcinoma; HepG2 cells; Triphala; tumor.

Resumen

Contexto: Triphala es una formulación tradicional a base de hierbas que consiste en frutos secos de tres plantas: Terminalia bellirica, Terminalia chebula y Phyllanthus emblica. Se utiliza tradicionalmente para tratar muchas dolencias, incluidos varios tipos de cáncer, promover la salud y la longevidad.

Objetivos: Evaluar la capacidad de un extracto acuoso de Triphala (TPL) para inhibir el crecimiento de células de carcinoma hepatocelular (HepG2) y en ratones.

Métodos: La actividad anticancerígena de TPL y sus extractos compuestos se evaluó in vitro mediante el ensayo MTT con células HepG2. Los ratones fueron inoculados con células HepG2 y luego divididos aleatoriamente en seis grupos (6 ratones/grupo): grupo de control, grupo de control positivo (intraperitoneal con 3 mg/kg de peso corporal de doxorubicina) y grupos de tratamiento con TPL (administración oral de 50, 100 y 200 mg/kg de peso corporal de TPL, respectivamente). Se determinó la actividad anticancerígena basada en el peso corporal, los volúmenes de crecimiento tumoral, el tiempo de supervivencia, el peso relativo de los órganos y los parámetros hematológicos tras administrar el TPL durante 14 días consecutivos.

Resultados: Los resultados demostraron que el extracto de TPL exhibió actividad antiproliferativa contra las células HepG2 de una manera dependiente del tiempo de tratamiento. El índice de selectividad (SI) mostró que TPL es altamente selectivo (SI>3) contra la línea celular HepG2 y no tiene actividad contra células no tumorales. La administración oral de extracto de TPL en dosis de 200 mg/kg de peso corporal inhibió el volumen de crecimiento tumoral y aumentó el tiempo de supervivencia. No se observaron anomalías en los parámetros hematológicos, el peso relativo de los órganos, el peso corporal y la morfología de los órganos.

Conclusiones: El extracto de TPL a las dosis de prueba fue un fármaco no tóxico. Nuestro hallazgo revela la eficacia anticancerígena del extracto de TPL.

Palabras Clave: actividad anticancerígena; carcinoma hepatocelular; células HepG2; Triphala; tumor.
INTRODUCTION

In Thai folk medicines, many herbal formulations consisting of two or more plant products are widely used for the treatment of various cancers in patients by rural practitioners. These folk medicines may contain active chemical constituents with multiple physiological and pharmacological activities and could be used in the treatment of various disease conditions. The discovery of effective herbs and elucidation of their underlying mechanism could lead to the development of an alternative and complementary method for cancer prevention and/or treatment.

Triphala is a traditional herbal formulation consisting of dried fruits from three plants namely, Terminalia bellirica (Gaertn.) Roxb. (Combretaceae), Terminalia chebula Retz. (Combretaceae) and Phyllanthus emblica L. (Phyllanthaceae) in equal formulation (1:1:1) (Peterson et al., 2017; Wongnopavich et al., 2009). Its traditional use is for treatment of many ailments, including various types of cancers, health promotion, and longevity. Triphala can be used to combine with the other folk medicine formula such as the combination with Ayurveda and showed hepatoprotective effect (Aswathy et al., 2019; Gupta et al., 2015; Patel et al., 2020).

Triphala extract was reported to have anticancer activity against several cancers both in vitro and in vivo such as breast cancer cell line, MCF-7 (Murthy, 2008; Sandhya et al., 2006), MDA-MB-231 (Cheriya mundath et al., 2018), cervical adenocarcinoma (Cheriyamundath et al., 2018), pancreatic adenocarcinoma, PANC-1 (Cheriya mundath et al., 2018; Shi et al., 2008) colorectal carcinoma cell lines (Wang et al., 2018), human gastric cancer cells (Tsering and Hu, 2018), hepatocellular carcinoma (Sahragard et al., 2021) and in transplantable mouse thymic lymphoma, barcl-95 (Sandhya et al., 2006).

The composite plant extracts also have anticancer activity. The T. chebula, T. bellirica, and P emblica extracts showed anticancer activity on cholangiocarcinoma cell lines by inducing the mitochondria apoptotic signaling pathway (Chekdaengphnanao et al., 2022). However, the antitumor potential in tumor-bearing mice of Triphala has not been clarified. The aim of this study was to evaluate the inhibitory effect of an aqueous extract Triphala on the growth of hepatocellular carcinoma cells (HepG2) in mice.

MATERIAL AND METHODS

Plant materials

The fresh fruits of T. bellerica, T. chebula and P. emblica were collected from the Mae Tam Reservoir, Lampang Province, Thailand in December 2019 (14O 57’14” N latitude, 102O 1’49” E longitude). Taxonomic authentication was identified by the Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. Voucher specimens of P. emblica, T. bellerica and T. chebula were preserved and deposited under voucher specimen numbers PBM-005668, PBM-005669 and PBM-005670, respectively at their herbarium (Nontakham et al., 2022). The fruits were cleaned and cut, and the seeds of each individual fruit were removed. It was dried, milled, and then mixed with these fruit powders in equal-proportion formulations (1:1:1).

Extract preparation

The powders (1 kg) of TPL, T. bellerica, T. chebula and P. emblica were separately refluxed with a hot water, filtrated, concentrated using a rotary evaporator and then lyophilized. The extracts were stored at -20°C. The extracts were freshly prepared in distilled water, sonicated and sterilized by using 0.4 µm syringe filter prior using in the experiments.

In vitro anticancer activity

Hepatocarcinoma cell line HepG2 cell line and African green monkey kidney-derived Vero cell line were cultured in MEM media supplemented with 10% fetal bovine serum, 100 µg/mL penicillin and 100 µg/mL streptomycin and incubated in a humidified atmosphere of 5% CO2 at 37°C. The cells (3 × 10^5 cells/well) were seeded into 96-well plate and then incubated for 24 h. The cells were treated with various concentration of each extract (1-1000 µg/mL). Doxorubicin (0.01-10 µg/mL) and distilled water were used as positive and negative controls. After incubation for 24, 48 and 72 h, the cells were analyzed for cell viability by using MTT assay (Siripong et al., 2012). Each concentration of drug was performed in six wells for three independent experiments. Cell viability was calculated and then expressed as 50% inhibitory concentration (IC50). The selectivity index (SI) was calculated by dividing the IC50 value into normal cells (Vero) by the IC50 value on cancerous hepatoma cells (HepG2) [1].

\[
\text{SI} = \frac{\text{IC50 of extract on normal cells (Vero)}}{\text{IC50 of extract on cancerous cells (HepG2)}}
\]

The SI value indicates the sample’s selectivity to the cell lines tested. Samples with a SI>3 was consid-

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ered to have a high selectivity for cancerous cells (Chothiphirat et al., 2019).

**In vivo experiments**

**Animals**

A total of 60 male SCID mice (20 ± 2 g, 5-week-old) were purchased from the Nomura Siam International Co., Ltd., Bangkok, Thailand. The mice were acclimatized for 1 week before performing the experiment. Mice were housed in polypropylene cages under standard laboratory conditions of the Animal laboratory of National Cancer Institute, Bangkok, Thailand (22 ± 2°C, 40-60% humidity with 12 h light/12 h dark cycle). The animals were fed with the standard pellet diet and filtered water ad libitum. The study protocol was approved by the Institutional Animal Care and Used Committee (IACUC) of the National Cancer Institute, Bangkok, Thailand (Protocol No. EC COA 041/2016).

**Cell preparation and implantation**

Human hepatocellular carcinoma HepG2 cells used for implantation were harvested when they were vigorous and were resuspended in PBS at a concentration of 1 × 10⁶ cells/mL. 200 µL of the prepared cell suspension was subcutaneously inoculated in the abdominal cavity of a SCID mouse.

**Animal treatment**

After implantation for 24 h, the mice (n = 12) were divided into 5 groups including: Group 1 was the hepatocellular carcinoma-bearing mice (control group) treated with 0.2 mL distilled water, per oral (po.). Group 2 was the hepatocellular carcinoma-bearing mice treated with doxorubicin 3 mg/kg, intraperitoneal (ip.). Groups 3, 4, and 5 were the hepatocellular carcinoma-bearing mice treated with TPL 50, 100, 200 mg/kg, po, respectively. The reference drug, extracts and vehicles were administrated for 14 consecutive days. Calculation of tumor volume was performed by using the formula 0.4 (a × b²), where a is the largest, and b, the smallest, diameter. The tumor volume thus calculated correlated well with the actual tumor weight (r = 0.980). Tumor growth inhibition (TGI) was defined as (1−T/C) × 100, where T indicates the mean tumor volume (mm³) of the test groups and C indicates the mean tumor volume (mm³) of the control group. Tumor size and body weight were determined every 2 days. On day 15, the mice (n = 6/group) were sacrificed, and their blood was collected. The hematological parameters, survival time and organ weights were analyzed. The remaining mice were measured for tumor volume until day 26.

**Statistical analysis**

Data were expressed as the mean ± standard deviation (S.D.). Differences between groups were evaluated by one-way ANOVA with the Dunnett’s multiple comparison test with SPSS statistical software version 23. Those in two groups were determined by using Student’s t-test. P<0.05 was considered statistically significant.

**RESULTS**

**In vitro anticancer activity**

The antiproliferative activity of Triphala and its individual constituents was evaluated using the MTT assay against HepG2 cancer cells and Vero non-cancerous cells. The findings of the study indicate that TPL and *T. bellirica* extracts demonstrated potent inhibitory effects against HepG2 cells. TPL exhibited considerable inhibition of HepG2 cells with IC₅₀ values of 259.17 ± 7.04 µg/mL (48 h) and 79.83 ± 3.16 µg/mL (72 h), while *T. bellirica* exhibited IC₅₀ values of 222.56 ± 23.40 µg/mL (48 h) and 71.88 ± 2.02 µg/mL (72 h) (Table 1). Furthermore, treatment with TPL and *T. bellirica* extracts for 72 h demonstrated no cytotoxicity towards the Vero cell line. The positive control drug, doxorubicin, exhibited strong cytotoxic effects on both HepG2 and Vero cells. Moreover, the selectivity index (SI) indicates the selective effects of the TPL against cancer cells versus non-cancerous cells (Chothiphirat et al., 2019). Also, TPL showed high selectivity (SI>3) against HepG2 cancerous cells at a 72 h incubation time (Table 1). Based on these results, TPL was selected for further investigation in an in vivo experiment.

**In vivo experiments**

The antitumor effect of TPL was analyzed in a tumor-bearing mouse model by transplanting HepG2 cancer cells into mice. As shown in Fig. 1A, tumor volume was reduced (significantly different from the control p<0.05) after oral administration of TPL at 100 mg/kg and 200 mg/kg. The tumor volume was measured daily until the 26th day. TPL’s tumor growth inhibition (TGI) was dose-dependently enhanced: 24.81% (50 mg/kg TPL), 30.76% (100 mg/kg TPL), 40.91% (200 mg/kg TPL). TPL produced similar effects on the tumor weight (Fig. 1B). Treatment with 200 mg/kg. TPL resulted in lower tumor weight (significantly different from the control p<0.05). However, TPL did not affect the body weight of mice of all groups (Fig. 2). These results suggested that TPL had specific antitumor effect on this animal model without adverse effect on the body weight. Moreover, the RBC, hemoglobin, hematocrit, WBC, lymphocytes,

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J Pharm Pharmacogn Res (2023) 11(3): 450
and platelet of mice in all TPL-treated groups were not different compared to the control group (Table 2). The mean survival times of the untreated group and the TPL-treated groups (50, 100, and 200 mg/kg) were 28, 30, 33, and 40 days, respectively. No abnormality of other hematological parameters, relative organ weight and morphology of organs were observed (data not show).

Table 1. Cytotoxicity and selectivity index (SI) of Triphala (TPL) and its composite extracts and doxorubicin on HepG2 and Vero cell lines at 24, 48, and 72 h of treatment.

<table>
<thead>
<tr>
<th>Extract/cell line</th>
<th>HepG2 24 h</th>
<th>Vero 24 h</th>
<th>HepG2 48 h</th>
<th>Vero 48 h</th>
<th>HepG2 72 h</th>
<th>Vero 72 h</th>
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<tr>
<td>TPL</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>259.17 ± 7.04</td>
<td>846.37 ± 10.04</td>
<td>79.83 ± 3.16</td>
<td>523.89 ± 9.54</td>
</tr>
<tr>
<td>T. chebula</td>
<td>&gt;1000</td>
<td>976.56 ± 21.67</td>
<td>780.88 ± 77.04</td>
<td>536.45 ± 6.72</td>
<td>235.70 ± 2.02</td>
<td>295.05 ± 5.63</td>
</tr>
<tr>
<td>T. bellirica</td>
<td>&gt;1000</td>
<td>858.94 ± 19.92</td>
<td>222.56 ± 23.40</td>
<td>475.96 ± 7.67</td>
<td>71.88 ± 2.02</td>
<td>178.45 ± 2.65</td>
</tr>
<tr>
<td>P. emblica</td>
<td>&gt;1000</td>
<td>821.58 ± 6.38</td>
<td>821.58 ± 6.38</td>
<td>496.56 ± 8.76</td>
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<tr>
<td>Doxorubicin</td>
<td>1.24 ± 0.10</td>
<td>0.55 ± 0.03</td>
<td>0.21 ± 0.04</td>
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</table>

The data are expressed as the means ± SD of three independent experiments. *SI value > 3 indicates high selectivity (Chothiphirat et al., 2019).

Table 2. Effect of Triphala (TPL) extract on hepatological parameters from hepatocellular carcinoma-bearing SCID mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Doxorubicin (3 mg/kg)</th>
<th>TPL (50 mg/kg)</th>
<th>TPL (100 mg/kg)</th>
<th>TPL (200 mg/kg)</th>
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<tr>
<td>RBC (&gt;10^6 cells/mm^3)</td>
<td>10.3 ± 0.3</td>
<td>11.0 ± 0.6</td>
<td>10.4 ± 0.9</td>
<td>11.7 ± 0.2</td>
<td>10.5 ± 0.5</td>
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<tr>
<td>Hemoglobin (g/dl.)</td>
<td>14.8 ± 0.8</td>
<td>15.5 ± 0.8</td>
<td>14.9 ± 1.1</td>
<td>15.3 ± 0.3</td>
<td>15.1 ± 0.7</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>49.0 ± 2.4</td>
<td>52.3 ± 2.0</td>
<td>49.3 ± 3.5</td>
<td>50.1 ± 1.1</td>
<td>49.1 ± 1.2</td>
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<tr>
<td>WBC (&gt;10^9 cells/mm^3)</td>
<td>1.0 ± 0.6</td>
<td>1.9 ± 1.2</td>
<td>1.57 ± 0.41</td>
<td>1.6 ± 0.4</td>
<td>1.8 ± 0.9</td>
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<td>Lymphocytes (%)</td>
<td>25.5 ± 9.2</td>
<td>35.0 ± 10.9</td>
<td>34.5 ± 10.9</td>
<td>35.0 ± 6.7</td>
<td>31.4 ± 9.3</td>
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<tr>
<td>Platelet (&gt;10^9 cells/mm^3)</td>
<td>7.5 ± 3.0</td>
<td>10.4 ± 3.2</td>
<td>10.6 ± 3.7</td>
<td>12.1 ± 2.8</td>
<td>14.7 ± 1.9*</td>
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<tr>
<td>SGPT (U/L)</td>
<td>135.0 ± 53.3</td>
<td>45.8 ± 14.4*</td>
<td>90.86 ± 40.02</td>
<td>35.4 ± 8.5*</td>
<td>34.4 ± 5.1*</td>
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<tr>
<td>SGOT (U/L)</td>
<td>806.5 ± 130.4</td>
<td>118.2 ± 51.0*</td>
<td>846.2 ± 126.9</td>
<td>123.8 ± 49.2*</td>
<td>151.2 ± 29.2*</td>
</tr>
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Values are means ± SD (n = 6). Comparisons between groups are made by one-way ANOVA, Dunnett’s multiple comparison test. *p<0.05 vs control.
DISCUSSION

Triphala is well-known traditional Ayurvedic formulation that has been used for centuries. It has been known to have anticancer property. This formulation consists of T. chebula, T. bellirica, and P. emblica fruits in equal proportion. Each plant extract also has several health benefits including anticancer activity both in vitro and in vivo. These plant extracts of Triphala formulation, especially P. emblica, contained high phenolic, flavonoid and tannic contents, exhibited inhibitory effect on inflammation, and showed strong anticancer activity against cholangiocarcinoma cells. While the T. bellirica extract was found to inhibit cell cycle arrest in the S phase. In addition, these plant extracts inhibited cholangiocarcinoma cell growth by inducing apoptosis via mitochondria apoptotic signaling pathway (Chekdaengphanao et al., 2022). In our study, the extracts of TPL and T. bellirica showed stronger antiproliferative activity on HepG2 growth than T. chebula, and P. emblica. The T. bellirica was reported to have antiproliferative activity against oral squamous cell carcinoma. Interestingly, the main compounds of this TPL were reported as gallic, chebulagic, ellagic, and chebulinic acids (Nontakham et al., 2022). Gallic acid was a bioactive compound found in T. bellirica extract, which contributed to free radical scavenging capacity and selective antiproliferative activity (Gupta et al., 2021). This ROS acted as the vital component in regulation of apoptosis and facilitated mitochondrial apoptosis to damage the DNA (Patra et al., 2020). The strong antiproliferative effect of TPL and T. bellirica extracts in our study might be the effect of gallic acid in the extracts.

Triphala was reported to have anticancer activity by modulating of multiple cell signaling pathways such as ERK, MAPK, NF-κB, Akt, c-Myc, VEGFR, mTOR, tubulin, p53, cyclin D1, anti-apoptotic and pro-apoptotic proteins (Prasad and Srivastava, 2020). Triphala inhibited tumor growth and migration of human gastric cancer cells both in vitro and in vivo in zebrafish xenograft model. The mechanism of antiproliferative and antimetastatic activities of Triphala on human gastric cancer cells was inhibition of phosphorylation of EGFR, Akt, and ERK (Tsering and Hu, 2018). Chebulinic acid was found to be a major component of Triphala and exerted potent effects of anti-

Figure 1. Suppressive effect of Triphala (TPL) extract on tumor growth and weight of hepatocellular carcinoma-bearing SCID mice. (A) tumor volume and (B) tumor weight.
Data are presented as the mean ± SD (n = 6), *p<0.05, **p<0.01, ***p<0.001 vs. control

Figure 2. Effect of Triphala extract on the body weight of hepatocellular carcinoma-bearing SCID mice.
Data are presented as the mean ± SD.
proliferation, pro-apoptosis, and antimigration of colorectal carcinoma cell lines through PI3K/AKT and MAPK/ERK pathways (Wang et al., 2018). Triphala extract was reported to have anticancer activity against breast cancer cell line, MCF-7. Anticancer effect of Triphala extract was demonstrated in a transplanted mouse thymic lymphoma, barcl-95, via apoptotic induction. In addition, Triphala at 40 mg/kg, significantly reduced tumor growth in mice transplanted with barcl-95 (Sandhya et al., 2006). Triphala was also reported to have inhibitory effect on the proliferation of HeLa (cervical adenocarcinoma), PANC-1 (pancreatic adenocarcinoma), and MDA-MB-231 (triple-negative breast carcinoma) cells by inducing apoptosis and interfering the reassembly of the microtubules (Cheriyamundath et al., 2018). These reports were correlated with our finding in this present study that Triphala could significantly suppress the HepG2 cell growth and the tumor growth in the hepatocarcinoma-bearing mice. These results confirmed the anticancer activity of Triphala extract. Moreover, Triphala is a safe formulation for oral administration. There were several reports to confirm the safety of Triphala using both acute and chronic toxicity tests in animal models and healthy volunteers (Arpornchayanon et al., 2022; Phetkate et al., 2019; 2020). Our present study also showed that Triphala aqueous extract had no toxicity at the tested concentration.

CONCLUSION

The present study showed that Triphala aqueous extract possesses anticancer effect when applied on the tested cells and in vivo model, using hepatocarcinoma-bearing mice. Therefore, Triphala could be considered a promising option as a complementary treatment in cancer patients.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

This research was supported by the Government Grant No. 4570 of the National Cancer Institute, Department of Medical Services, Ministry of Public Health Bangkok, Thailand. Authors also thank Dr. Pongpun Siripong, Senior scientist at National Cancer Institute Thailand, for her kind suggestions.

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Anticancer effect of Triphala extract in mice

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<th>Suthamnatpong N</th>
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