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Cancer Cytotoxic and Anti-HIV Potential of Triphala Herb Mixture on from Chae Son, Lampang, Thailand

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Authors' contributions

This work was carried out in collaboration among all authors. Author WP designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors WB and AC managed the analyses of the study. Authors KS, JL and CN managed the analyses of anti-HIVs assay and cytotoxicity. Authors BW, PS and PU managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: The objective of this study was to compare the stability properties of the two years for the quality of Tri-Pha-La Herbal Drink, Thai traditional and Ayurvedic medicine. This study aimed to compare the anticancer and anti-HIV efficacy of the solvent extracts and gallic acid of the three fruits. The research was done between A.D. 2017 to A.D. 2018.
Materials and Methods: In the present study, the cancer cell lines, KB, HT 29, MCF-7, A 549, CL and ASK treated with Triphala fruits extract at concentrations of 4 mg/mL for 72 h except 48 for P-

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388) . The cytotoxic activity was expressed as 50% effective dose (ED_{50}). In addition, anti-HIV activity of the all Triphala fruits solvent extracts was performed by anti-HIVs-1RT. The tested extracts were prescreened at 200 µg/mL and only those which were very active (≥70% inhibition) at this concentration were further determined for the dose, µM, that inhibited 50% HIV-1 RT activity (IC_{50}). Moreover, anti-syncytium (MC99+1A2) was performed by the Triphala fruits extract. The results were expressed as the concentration that inhibited 50% formazan formation in uninfected cells (IC_{50}). The therapeutic index (TI) was calculated using the equation: $TI=IC_{50}/EC_{50}$.

Results: The results were compared between years A.D.2017/2018. Amongst six ethyl acetate and methanol extracts (A.D. 2017) showed less than 20 *µ*g/mL cell viability in P-388 cell lines. However, the four ethyl acetate and methanol extracts (A.D. 2018) were also inhibited P-388 cell species. Furthermore, each gallic acid, isolated from three fruits.

Conclusion: Triphala (A.D.2017/2018) were also strongly inhibited to P-388 cell species. Anti-HIV-RT activity of the all Triphala fruits solvent extracts (A.D. 2017) except hexane extracts showed very active which is consistent with A.D. 2018. *P. emblica*-ethyl acetate extract (A.D. 2018) was the most promising anti-syncytium (MC99+1A2) among evaluated all three fruits Triphala extracts: it afforded the lowest EC_{50} 8.85 µM and therapeutic index 2.10. The results evidently showed the capacity of Triphala as potential chemopreventive and anticancer drug and immunity.

Keywords: Triphala; Terminalia chebula; Terminalia bellirica; Phyllanthus emblica; cytotoxicity; anti-HIV.

1. INTRODUCTION

There is a gathering of farmers in Chae Son, Mueang Pan, Lampang, Thailand. Triphala fruits are abundant natural resources in the district, there are usage herbal drinking water. The study on the properties of Triphala fruits composition before specifying the properties of the products for use as a label that matches the product. Triphala is very important natural ayurvedic formulation, commonly prescribed by most healthcare practitioners in Thailand. It is an anequiproportional mixture of dried fruits of three medicinal herbs, *Phyllanthus emblica* Linn. (PE), *Terminalia bellirica* (Gaertn) Roxb. (TB), *Terminalia chebula* Retz. (TC), also known as the 'Triphala'. The proportion of 1:1:1 or even 1:1:2, each of which consists of a variety of structurally varied chemicals [1]. Triphala is traditionally been used as laxative in chronic constipation, colon cleansing, digestion problems and poor food assimilation [2,3]. It has also been used in cardiovascular disease, high blood pressure disease, serum cholesterol reduction, poor liver function, large intestine inflammation, and ulcerative colitis [4]. Triphala promotes immunomodulatory system and helps in improving the body's defence system [5-9]. It has also shown to inhibit the growth of carcinogen induced stomach cancer, thymic lymphoma, and pancreatic cancer in mice [10,11]. Based on several scientific studies, Triphala, in the equal proportion formulation (1:1:1), has demonstrated many effects in mice, that is, chemoprotection, anti-inflammation, and immunomodulation [12,13]. The anticancer activity of Triphala has been investigated. It has also been found in inhibiting growth of several malignancies including both in *vitro* and in *vivo*, such as prostate cancer, breast cancer, and pancreatic tumor [14-16]. The results of pharmaceutical analyses revealed that triphala is rich in terpenoids, saponins, tannins, phenolic acids and flavonoids [17]. Among these compounds, tannin-related compounds, and ellagitannins, especially ellagic acid, gallic acid, chebulinic acid and chebulagic acid, are considered the major constituents of the bioactivities [18–20]. In this study, information on compound and extracts in herbal medicines of triphala formula was acquired from Chae Son National Park, Lampang, a Province of Thailand, in January, 2017 and 2018. With these multiple medicinal properties, especially cytotoxicity and anti-HIVs. The research objectives of this were to comparing those stability properties of the two years for the quality of Tri-Pha-La Herbal Drink. This is a problem solving of poverty and strengthen the economic foundation of farmers to be strong and sustainable economy.

2. MATERIALS AND MATHODS

2.1 Plant Material

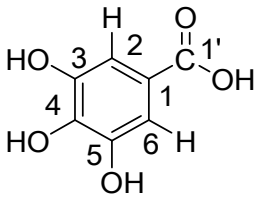
Triphala is an ayurvedic herbal formulation of dried fruits from three herbal plants in 2:1:1 propertions: *Phyllanthus emblica* (BKF 113645), *Terminalia chebula* (BKF 170476) and *Terminalia bellirica* (BKF 188656). These fruits were

collected from Chae Son National Park, Lampang, a province of Thailand, in January, 2017 and 2018 by Mr. Narong Nuntasaen. The plants were identified and deposited in the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry of National Resources and Environment, Bangkok, Thailand.

2.2 Extraction and Isolation

Dried three fruits of *P. emblica* (2 kg), *T. chebula* (2 kg) and *T. bellirica* (2 kg) and Triphala herbal in 2:1:1 propertions: *P. emblica* (2 kg), *T. chebula* (1 kg) and *T. bellirica* (1 kg) were ground into powder and then extracted successively with hexane, ethyl acetate and methanol for 5 times each (5×7 L). Removal of solvents from each extract under reduced pressure afforded *P. emblica* (crude hexane ,33.55 g, crude ethyl acetate, 72.04 g and crude methanol, 622.02 g) extracts; *T. chebula* (crude hexane, 24.0 g, crude ethyl acetate, 61.0 g) and crude methanol, 1083 g) extracts; *T. bellirica* (crude hexane ,63.83 g, crude ethyl acetate, 35.42 g and crude methanol, 571.43 g) and Triphala herbal crude methanol, 1200 g, respectively. The crude ethyl acetate extract of each three fruits and triphla extract were selected to separate by flash Column Chromatography (CC) over silica gel eluted with gradient systems of ethyl acetate:hexane and methanol: ethyl acetate, to give the brown precipitate which was filtered out and then recrystallized from ethanol all of extracts to yield same gallic acid.

2.3 Physical Properties of Gallic Acid



White needles, m.p. 259.2 – 260.52ºC; IR (KBr) cm^{-1}: 3300 (OH), 1701 (C=O), 1623, 1541, 1452 (C=C), 1151(C-O-C); EIMS *m/z*: 170.12 ($[M]^+$, $C_7H_6O_5$), 153(100), 79(10) ^{1}H NMR (400 MHz, CD_3OD): δ 7.08 (1H, d, *J* = 2 Hz, H-2), 7.08 (1H, d, *J* = 2 Hz), 9.12 (1H, s, COOH). ^{13}C NMR (100 MHz, CD_3OD): δ 138.18 (C-1), 109.0 (C-2), 144.98 (C-3-OH), 120.62 (C-4-OH), 144.98 (C-5-OH), 109.0 (C-6), 168.98 (C-1′). HMBC correlations, H/C: 2/4, 3, 2, 1, 1′; 6/4, 5, 6, 1, 1′. COSY correlations, H/H: 2/6.

2.4 Cytotoxicity Assay

Cytotoxicity assay of the tested extracts were carried out using the in *vitro* sulforhodamine B (SRB) method [21] and used ellipticine as a positive control. Test samples were dissolved in DMSO as a concentration at 4 mg/mL and were increased triplicate until the final concentration at 0.1%. The cancer cell species were grown in a 96-well culture plate as follows: P-388, in RPMI-1640 with 5% fetal bovine serum (FBS). The KB, Col-2, MCF-7 and ASK cell lines were cultured in MEM (minimum essential medium with Earle's salt and L-glutamine) with 10% FBS, while Lu-1 was grown in MEM with 5% FBS. The cultures were then added with extracts and compound and incubated in 37°C for 72 h (48 h for P-388) with 5% CO_2 in the air, and 100%. Cells were fixed with a final concentration of 10% trichloroacetic acid and stained with 0.4% sulforhodamine B in 1% acetic acid. The bound and dried stain were solubilized with 10 mM trizma base, after removal of the unbound dye by washing. The absorbance at wavelength 510 nm was read on a Fluostar optima BMG plate reader. The cytotoxic activity was expressed as 50% effective dose (ED_{50}).

2.4 Anti-HIVs Assay

Anti-HIV-1 reverse transcriptase assay: Anti-HIV-1 reverse transcriptase assay extracts were dissolved in DMSO at the concentration of 20 mg/mL and processed to remove tannin [22]. The assay was carried out in duplicate in a 96-well culture plate using tannin-free supernatant of the extracts as previously described [23]. An appropriate amount of HIV-1 reverse transcriptase (Amersham Pharmacia Biotech Asia Pacific Ltd., Hong Kong) was standardized with fagaronine chloride. This extracts and nevirapine were used as positive controls, while DMSO without the extracts as a negative control. Test extracts were prescreened at 200 µg/mL and only those which were very active (≥70% inhibition) at this concentration were further determined for the dose, µM, that inhibited 50% HIV-1 RT activity (IC_{50}).

Cell-based assay for anti-HIV-1: The syncytium assay was carried out in triplicate using ΔTat/revMC99 virus and 1A2 cell system [24,25], starting at the final concentrations of 3.9–125 µg/mL or higher. Virus control and cell control wells contained neither the extracts nor virus; cytotoxicity control wells containing cells with the extracts and a positive control, i.e., azidothymidine, AZT, were included. The result

was expressed as 50% effective concentration (EC_{50}). Cytotoxicity of the extracts was also carried out, in parallel and in duplicate, using colorimetric XTT assay. The result was expressed as the concentration that inhibited 50% formazan formation in uninfected cells (IC_{50}). The therapeutic index (TI) was calculated using the equation: $TI=IC_{50}/EC_{50}$.

3. RESULTS AND DISCUSSION

The significance in the pharmacological effects of bioactive compounds or extracts of cancer treatments and prevention has been increased dramatically over the past twenty years. It has been shown many anti-cancer activities in various cancer cells in Table 1. The investigation on toxicity which compared fruits of the three plants and Triphala (A.D.17/A.D.18) on P-388, KB, HT 29, MCF-7, A 549, CL and ASK cell showed significantly potential on P-388 (A.D.17); *T. chebula*-ethyl acetate extract(10.27 μg/mL), *T. bellirica*- ethyl acetate extract (13.12 μg/mL), *T. bellirica*- methanol extract(12.81 μg/mL), *P. emblica*-ethyl acetate extract (14.21 μg/mL*), P. emblica*- methanol extract(14.21 μg/mL) and Triphala-methanol extract (13.55 μg/mL). In accordance to A.D.17, the results in A.D.18 also found the toxicities against P-388 cancer cells: *T. chebula*-ethyl acetate extract (10.57 μg/mL), *T. bellirica*- ethyl acetate extract (12.38 μg/mL), *T. bellirica*- methanol extract(12.81 μg/mL) and *P. emblica*-ethyl acetate extract (3.3 μg/mL), except *P. emblica*- methanol extract. However, Triphala-methanol dis not inhibit the proliferation of the cancer cells. The American National Cancer Institute guidelines (NCI) determine the limit of crude extracts at 50% (ED_{50}) and inhibited proliferation less than 20 μg/mL [26]. ED_{50} values below this stringent point were observed with six extracts (A.D.17) and four extracts (A.D.18). Triphala becomes one of the highly potential herbal medicines in cancer treatment and prevention because all of three triphala compositions with outstanding anticancer properties [27]. There are many studies showing the anticancer of *P. emblica* [28,29]. In the experiment, *P. emblica* fruit extract inhibited the spread of many types of cancer cells [28]. The underlying mechanism by which *P. emblica* inhibits cancer cells is still not clear. There are many possible ways of dealing with cell cycle interference [29]. However*, T. chebula*, one of the composition of triphala, found to have an effect on the toxicity of cancer cells [30,31]. There are a very limited number of studies on the evaluation of cytotoxicity of *T. bellirica* fruit. The IC_{50} value of a hydroglycol extract of *T. bellirica* fruit against normal mouse fibroblast cells and mouse melanoma cells were 5.43 mg/mL and 2.0 mg/mL at 48 h incubation, respectively [32]. In current study, we showed the cytotoxicities of the solvent extracts; *P. emblica*, *T. bellirica* and *T. chebula* together with Triphala, in A.D.17 to A.D. 18. The result varied with season of harvesting and environment. Based on the obtained results, the cytotoxicity tested in A.D.17 showed better anti P-388 cell line activity than in A.D.18. According to previous literatures, no report the effect of annual variation on the cytotoxicities to P-388 cell lines. Moreover, we considered all the cytotoxicities in solvent extracts and compounds. The best anti P-388 effect of the extracts and compound were found in both two study years. (Table1). Further research, requires additional determine the activity mechanism of Triphala fruits. Finally, the action of solvent extracts and stability definitely required for pharmacological application of Triphala herbal drink as anticancer agents for cancer therapy.

The HIV reverse transcriptase enzyme inhibition due to each solvent extracts were determined using HIV-RT inhibition assay by using of HIV-1 reverse transcriptase assay kit (Amersham Pharmacia Biotech Asia Pacific Ltd., Hong Kong). The RT activity on inhibiting of three fruits and triphala solvent extracts were analyzed. Inhibitions of HIV-RT by solvent extracts were presented in Table 2. The seven ethyl acetate and methanol extracts (A.D.17) showed the highest inhibition (>70%, VA, very active) of recombinant HIV-RT ; *T. chebula*-ethyl acetate extract (86.86%), *T. chebula*-methanol extract (90.90%), *T. bellirica*- ethyl acetate extract (101.98%), *T. bellirica*- methanol extract (106.44%), *P. emblica*-ethyl acetate extract (85.11%), *P. emblica*- methanol extract (91.02%) and Triphala-methanol (97.00%) at 200 μg/mL. The seven ethyl acetate and methanol extracts (A.D.18) also displayed the highest inhibition (>70%, VA, very active) of HIV-RT; *T. chebula*-ethyl acetate extract (90.02%), *T. chebula*-methanol extract (87.72%), *T. bellirica*- ethyl acetate extract (96.60%), *T. bellirica*- methanol extract (90.74%), *P. emblica*-ethyl acetate extract (96.83%), *P. emblica*- methanol extract (92.23%) and Triphala-methanol (88.82%) at 200 μg/mL. Previously, *P. emblica* had shown the same effect on inhibiting HIV-RT [33]. Part of *P. emblica* plant extract was shown the highest inhibition on recombinant HIV-RT (91% and 89%, respectively) at 1 mg/mL concentration. In

Table 1. Cytotoxicities of extracts and pure compound of A.D. 2017 to A.D. 2018

Extract	*Cell line (μg/mL), A.D. 2017							Extract	Cell line (μg/mL), A.D. 2018						
	*P-388	KB	HT 29	MCF-7	A 549	CL	ASK		*P-388	KB	HT 29	MCF-7	A 549	CL	ASK
Ellipticine	0.48	0.51	0.57	0.48	0.50	0.61	0.50	Ellipticine	07.46	0.53	0.76	0.54	0.54	0.60	0.53
T. chebula-hexane extract	-	-	-	-	-	-	-	*T. chebula*-hexane extract	-	-	-	-	-	-	-
T. chebula-ethyl acetate extract	10.27	-	-	-	-	-	-	*T. chebula*-ethyl acetate extract	10.57	-	-	-	-	-	-
T. chebula-methanol extract	-	-	-	-	-	-	-	*T. chebula*-methanol extract	-	-	-	-	-	-	-
T. bellirica-hexane extract	-	-	-	-	-	-	-	*T. bellirica*-hexane extract	-	-	-	-	-	-	-
T. bellirica- ethyl acetate extract	13.12	-	-	-	-	-	-	*T. bellirica*- ethyl acetate extract	12.38	-	-	-	-	-	-
T. bellirica- methanol extract	12.81	-	-	-	-	-	-	*T. bellirica*- methanol extract	12.81	-	-	-	-	-	-
P. emblica-hexane extract	-	-	-	19.19	-	-	-	*P. emblica*-hexane extract	-	-	-	-	-	-	-
P. emblica-ethyl acetate extract	12.25	-	-	-	-	-	-	*P. emblica*-ethyl acetate extract	15.3	-	-	-	-	15.27	15.39
P. emblica-methanol extract	14.21	-	-	-	-	-	-	*P. emblica*-methanol extract	-	-	-	-	-	-	-
Triphala-methanol	13.55	-	-	-	-	-	-	Triphala-methanol	-	-	-	-	-	-	-
Gallic acid from *T. chebula*	2.68	-	-	-	-	11.99	12.98	Gallic acid from *T. chebula*	2.96	-	-	-	-	10.59	15.1
Gallic acid from *T. bellirica*	2.45	-	-	-	17.13	11.04	11.73	Gallic acid from *T. bellirica*	2.58	-	-	-	16.32	11.99	12.36
Gallic acid from *P. emblica*	1.79	-	-	-	13.63	9.2	10.3	Gallic acid from *P. emblica*	1.86	-	-	-	14.2	8.65	11.1

** Cytotoxic assay: ED_{50} less than 20 μg/mL were considered active for extracts and less than 4 μg/mL for pure compound, *P-388: murine lymphocytic leukemia, KB: human epidermoid carcinoma, HT 29: human colon adenocarcinoma, MCF-7: human breast cancer, A 549: human lung adenocarcinoma, CL: Chang Liver, ASK: rat glioma*

Table 2. Anti-HIVs of extracts in A.D. 2017 to A.D. 2018 as determined by using cell based, reverse transcriptase, $^{\Delta Tat/Rev}$MC99 virus and 1A2 cell line assays

Extract	A.D. 2017						Extract	A.D. 2018					
	Anti-HIVs-1RT		Anti-syncytium (MC99+1A2)					Anti-HIVs-1RT		Anti-syncytium (MC99+1A2)			
	%inhibition	Activity	IC_{50}	EC_{50}	TI	Activity		%inhibiti	Activity	IC_{50}	EC_{50}	TI	Activity
T. chebula-hexane extract	45.44	WA	104.69	88.39	1.18	Active	*T. chebula*-hexane extract	29.31	I	230.59	25.91	8.90	Active
T. chebula-ethyl acetate extract	86.86	VA	55.98	37.54	1.49	Active	*T. chebula*-ethyl acetate extract	90.02	VA	58.33	16.80	3.47	Active
T. chebula-methanol extract	90.90	VA	116.22	26.20	4.43	Active	*T. chebula*-methanol extract	87.72	VA	62.68	16.84	3.72	Active
T. bellirica-hexane extract	-7.67	I	<7.80	<7.80	-	Toxic	*T. bellirica*-hexane extract	11.58	I	<8.90	<8.90	-	Toxic
T. bellirica- ethyl acetate	101.98	VA	116.92	12.42	9.41	Active	*T. bellirica*- ethyl acetate	96.60	VA	35.49	17.83	1.99	Active
T. bellirica- methanol extract	106.44	VA	107.10	12.26	8.73	Active	*T. bellirica*- methanol extract	90.74	VA	31.22	17.43	1.79	Active
P. emblica-hexane extract	-11.21	I	215.12	45.68	4.71	Active	*P. emblica*-hexane extract	20.22	I	208.65	35.53	5.87	Active
P. emblica-ethyl acetate extract	85.11	VA	38.86	9.20	4.22	Active	*P. emblica*-ethyl acetate extract	96.83	VA	19.52	8.85	2.10	Active
P. emblica- methanol extract	91.02	VA	32.10	10.69	3.00	Active	*P. emblica*- methanol extract	92.23	VA	48.08	13.72	3.50	Active
Triphala-methanol	97.00	VA	75.59	12.44	6.07	Active	Triphala-methanol	88.82	VA	51.54	16.38	3.15	Active
AZT	-	-	$>10^{-8}$	$5x10^{9}$	>2	-	AZT	-	-	$>10^{-8}$	$5.11x10^{-9}$	>2	-

** Anti-HIV-1RT activity express as % inhibition at 200 µg/mL (radioactive) or 667 µg/mL (non-radioactive): very active (VA) = >70% inhibition, moderately active (MA) = 50% to 69% inhibition, weakly active (WA) = 30% to 50% inhibition and inactive (I) = <30% inhibition*

addition, *T. chebula* and *T. bellerica* showed significant inhibitory effect on HIV-RT with IC_{50} < or = 50 micrograms/mL [34].

4. CONCLUSION

Triphala, a treaditional Ayurvedic formulation, contains of the dried fruits of three plants, *T. chebula*, *T. bellirica* and *P. emblica*. They recurrently use in many folk medicines. The herbal formulation posses several pharmacological activities including anticancer and anti-HIV. The cytotoxic effects of the three fruits and Triphala (in A.D.17/18) were mostly against P-388 cancer cells. Furthermore, the extracts (in A.D.17/18) were also potent with anti-HIV both of two mechanism; anti-HIVs-1RT and anti-syncytium (MC99+1A2). Therefore, these results evidently showed the capacity of Triphala as a potentially chemoprevention and anticancer drug and immunity.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All protocols involving cell experiments were approved by the cell Ethics Committee of Lampang Rajabhat University, Lampang, Thailand.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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