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Superoxide Anion Scavenging Activity and Mitochondrial toxicity of Various Tamarind (*Tamarindus indica* L.) Seed Coat Extracts

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Abstract

Introduction: Current research concentrates on pharmaceuticals that emphasis with compound from natural products or their derivatives. Tamarind seed coat is also well known in health benefits such as anti-oxidant activity, anti-inflammation and anti-diabetic but anti-cancer is rarely. Thus, this study was to investigate the extraction condition and effects on cellular toxicity of Tamarind seed coat. **Methods:** Extractions with super critical CO_2 with ethanol co-solvent as well as with ethanol and water extraction were carried out. The effects of Tamarind seed coat extracts on superoxide anion scaveng ing activity and mitochondrial toxicity were determined. The supercritical extraction was performing with various ethanol co-solvent. The solvent extracts were obtained from 50 and 95% ethanol and water used as a polar phase of extraction. Seven extracts were obtained and free radical scavenging activ ity was determined by superoxide dismutase (SOD)-like activity assay. The mitochondrial toxicity was analyzed by mitochondrial dehydrogenase activity (WST assay) and mitochondrial membrane potential (MMP assay). **Results:** All extracts exhibited high potential of superoxide anion scavenging activity over the IC50 range <5-25 µg/ml. WST assay was analyzed on various cells culture as well as Caco-2, HepG2, Hela, L929 and HK-2. The MMP assay was further investigated on Hela cells by fluorescence technique. Interestingly, the tamarind seed coat with water extract showed the highest toxicity to cancer cells but not to normal cells. The IC50 of water extract that exhibited on Caco-2, HepG2 andHela cells were 99.86, 81.15 and 15.06 µg/ml, respectively. The IC50 on normal cells (L929 and HK-2) showed at 444.16 and over 1,000 µg/ml. The toxicity of water extract on mitochondrial membrane was strongly as valinomycin (chemotherapeutic agent). **Conclusion:** From our findings it was conclude that tamarind seed coat extract from water may have strong toxic effect on cancer cells via mitocho ndrial membrane degradation while no toxic effect on normal cells.

Keywords: Tamarind seed coat, supercritical extraction, superoxide dismutase, cytotoxicity, mitochondrial membrane potential

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1. Introduction

Tamarind (*Tamarindus indica* L.) belongs to Fabaceae and grows naturally in many tropical and subtropical. Tamarind is an important plant resource for food materials. The flower and leaf are eaten as vegetables; the germ obtained from the seed is used for manufacturing tamarind gum which is well-known in jellose. Tamarind also contains antioxidants which are useful in the food, pharmaceutical and cosmetics industries (Siddhuraju et al., 2007). Moreover, the seed has well balanced essential amino acids, cystine and methionine and the kernel protein was rich in lysine, glutamic acid, aspartic acid, glycine, and leucine and the in-vitro digestibility was 71.3%(Bhattacharya et al., 1994).Tamarind seeds are also reported to contain phenolic antioxidants, such as 2-hydroxy-3, 4-dihydroxyacetophenone, methyl 3,4-dihydroxybenzoate, 3,4- dihydroxyphenyl acetate and epicatechin and exhibit antioxidant potential by reducing lipid peroxidation *in vitro* (Tsuda et al., 1994). The fruits and seeds of this plant showed anti-bacterial, anti-inflammatory and anti-diabetic effects (Paula et al., 2009 and De et al., 1999). The aqueous extract of tamarind seeds coat was found to have potent anti-diabetic and anti-hyperlipidemic activities that reduce blood sugar level and total cholesterol and triglycerides, respectively(Maiti et al., 2004 and Maiti et al., 2005).

Most research on Tamarind has concentrated on the fruits and seeds of this plant, mainly extracted using polar solvents. Although, solvent extraction is an easy and convenient separation method that can be carried out at ambient conditions the toxic effects of the solvent residue must be taken into consideration. Supercritical fluid extraction using CO_2 is considered to be more desirable since no toxic solvent residue remains. The extraction using supercritical fluid extraction with CO_2 (SFE), supercritical fluid extraction with a 10% ethanol co-solvent (SFE-EtOH) and solvent extraction using ethyl acetate (SX-EtAc) are found that SFE are unable to extract significant quantities of antioxidants and that (-)-epicatechin, using of SFE-EtOH provide superior extraction results as well as good antioxidant activity as measure using peroxide value on lard(Tsuda et al., 1994). It had been reported that the extraction of (-)-epicatechin with pure CO_2 was very low, (22 μg of (-)-epicatechin per 100 g of seed coat. The use of a 10% ethanol co-solvent resulted in a much higher yield of (-)-epicatechin, (13 mg/100 g), under the best conditions which were found to be 40°C and 10 MPa. Solvent extraction using ethanol has a higher selectivity than ethyl acetate for extraction of (-)-epicatechin; yields of (-)-epicatechin using ethanol were about 150 mg/100 g (Luengthanaphol et al., 2004).

Even though the seed coat possesses antioxidant activity and other health beneficial effects, the cellular toxicity of Tamarind seed coat extract remains unexplored. The identification of cytotoxic effects is a critical element of the small molecule drug discovery process. Equally important is the ability to automate as many aspects of the drug discovery process as possible. Cytotoxic effects of molecules are often first observed as perturbations of normal cellular

organelle functionality. The mitochondrial membrane potential provides much of the cell's energy needs and as such is very tightly regulated. In the intrinsic pathwayof apoptosis, mitochondria play a key role. Mitochondrial permeability transition, during which the electro chemical gradient across the mitochondrial membrane is lost, is a key step in the induction of apoptosis. Mitochondriatransmit cell death signals to the cytosol, leading to theactivation of caspases. Thus the ability to assess mitochon drial membrane potential can provide information regarding apoptotic degradation and cell death. Therefore, the present study was aimed on evaluating their free radical scavenging capacity and mitochondrial toxicity of different extracts from Tamarind seed coat samples. This results in the eventual execution.

2. Materials and Methods

Extraction condition

The tamarind seeds from sweet Thai tamarind were purchased from a local market in Bangkok. The seed coats were mechanically separated from the germ. Seeds were grounded using mechanical grinder several times until it became homogenous powder. The supercritical fluid extractions with CO_2 (SFE) and the super critical fluid extractions with CO_2 plus 10, 20, 50, 95% ethanol co-solvent (SFE-EtOH) were carried out at 37^{o}C and 30MPa. The flow rate of super critical CO_2 with co-solvents were set at 2.5 ml/ min. The solvent extraction was extracted by stirring with 50 and 95% ethanol for 48 h and filtering through Whatman No. 1 filter paper.The solvent of extracts were evaporated under reduced pressure (34–36 kPa) using a rotary vacuum-evaporator at 40^{o}C and the contents were freeze-dried, respectively. The water extraction was performing similar as solvent extraction. The freeze-dried extracts thus obtained were used directly for the assessment of SOD-like activity and mitochondrial toxicity assays.

Determination of superoxide anion ($O_2^{\bullet-}$) scavenging activity

Scavenging activity is based on the reduction rate of $O_2^{\bullet-}$ by superoxide dismutase (SOD). The $O_2^{\bullet-}$ were generated in the system by xanthine oxidase (XO) activity. The water-soluble tetrazolium salt produced a water-soluble forma zan dye up on reduction with a superoxide anion. The rate of the reduction with $O_2^{\bullet-}$ are linearly related to the XO activity, and is inhibited by SOD. Therefore, inhibition activity of SOD or SOD-like materials can be determined by a colorimetric method.Seed coat extracts was added (20 µl) to each well sample and blank of 96 well plate and mixed with 200 µl of working solution and followed by adding 20 µl of enzyme working solution to each sample and blank. The wells were mixed thoroughly and incubated at 37°C for 20 min. After incubation, the absorbance was determined at 440 nm. The SOD activity was express by rate of inhibition (%).

Determination of mitochondrial toxicity

Cell culture and reagent

The various cells line was used in experiment including cancer and normal cells.

Human colonadenocarcinoma (Caco-2) cells, Human liver hepatocellular carcinoma (HepG2) cells, Cervix adinocarcinoma (Hela) cells,Human kidney proximal tubular (HK-2) cells, Mouse subcutaneous connective tissue (L929) cells line were used to determine mitochondrial toxicity. All cells were grown in DMEM medium and supple ment with 10% (v:v) of fetal bovine serum except for L929 was grown with MEM medium and supplement with 10% (v:v) of horse serum in a humidified incubator at 37°C and 5% CO_2.

Mitochondrial dehydrogenase activity assay (WST assay)

This assay depends on the cleavage of the soluble tetrazolium salt (WST) to form water soluble formazan via the action of mitochondrial succinate dehydrogenase found in visible cells. Cells were seeded in 96 wellplates and culture for 24 h in 5% CO_2 incubator at 37°C prior to treatment with various concentrations of Tamarind seed coat extracts. After 24 h treatment, the medium in each well was replacing with 100 µl of new medium and add 10 µl of WST solution. Cells were incubated in a 5% CO_2 incubator at 37°C for 30 min. Cells viability was quantified by spectrophotometer at a measuring wavelength of 450 nm.

Mitochondrial membrane potential (MMP assay)

The MMP assay was analyzed by fluorescence method. Mitochondrial membrane potential can be use to detect apoptosis, it analyzed by 5,5',6,6'-Tetrachloro-1,1',3,3'-tetrae-thylbenzimidazolylcarbocyanine iodide (JC-1), cationic fluorescence dye. JC-1 dye accumulates throughout the cell's cytosol as a monomer, emitting primarily green fluorescence. In healthy, energized cells, the mitochondria's negative charge established with an intact membrane potential allows the lypophilic dye to enter the mitochondrial matrix and accumulate. The increasing of mitochondrial matrix concentration dye forms J-aggregates that exhibit red/orange fluorescence. Mitochondrial damage or loss of membrane potential, the dye cannot accumulate in the mitochondria, which is indicated by a lack of orange fluorescence. Hela cells at $1x10^5$were grown and incubate with 5, 15 µg/ml of Tamarind seed coat water extract for 24 h. Valinomycin was use as positive control, 0.5 µg/ml incubate with Hela cells for 4 h. The extract was remove and incubatedtrysinized cells with JC-1 dye solution at 37°C and 5% $CO_2$for 30 min. After remove dye, cells were washing by PBS and resuspened before analyzed by using high content imaging fluorescence microscope (INCellAnalyzer 2200, GE HealthCare). The generated fluorescent products can be visualized using a double-field fluorescence with fluorescein isothiocyanate (FITC, 475/512 nm) and Texas Red (575/620 nm) filters. Fluorescent intensity was analyzed using the software IN Cell Developer Toolbox V 1.9.1. The ratio of red fluorescent intensity and green fluorescent intensity was calculated and presented as the mean+SD.

3. Results and Discussion

Tamarind seed coat extracts

Table 1 showed the extract yield of the various tamarind seed coat extraction condition range from 6-50% of dry weight. These results showed that the extraction yields were increase by increasing percentage of co-solvent TSC331 (10%), TSC332 (20%), TSC3350 (50%) and TSC3395 (95%)). S Luengthanaphol et al., 2004 showed that the extraction by supercritical CO_2 without co-solvent showed lower of (-)-epicatechin than ethanol co-solvent extraction. Because ethanol is a polar molecule, the polar compound will easily dissolve in the ethanol solvent. Therefore SFE experiments with the additional of ethanol as a co-modifier were performed. However solvents extract including 50 (TSC50) and 95% (TSC95) ethanol showed higher yield content more SFE with co-solvents. Interestingly, water extraction was the best solution for extraction. The highest extraction yield was found in tamarind seed coat extract by water (TSCH). The resulted similar withNakchat et al., 2014that showed TSCE-W (water extract), consisting of the highest phenolic content much higher than TSCE-E (70% ethanol). The temperature and pressure, was also observed by Luengthanaphol et al., 2004 they showed highest of (-)-epicatechin content in the condition of 35°C and 30MPa.

Table 1: Yield (%) of tamarind seed coat extracted by supercritical fluid extraction with co-solvents, solvent extraction and water extraction

Samples	Pressure (MPa)	Temperature (ºC)	Co-solvent (%EtOH)	Yield (%) (w/w)
TSC331	30	30	10	6.472
TSC332	30	30	20	7.737
TSC3350	30	30	50	17.018
TSC3395	30	30	100	12.928
TSC50	solvent extract		50	19.809
TSC95			95	49.242
TSCH	water extract		water	53.879

Superoxide anion ($O_2^{\bullet-}$) scavenging activity

The scavenging activity of the extracts depicted a dose-response (Table 2). All of extract showed strong $O_2^{\bullet-}$ scavenging activity in range of IC50 values 5-17 µg/ml. The inhibition reaction appeared still occurring more than 50%, even at the dose less than 5 µg/ml of four extracts (TSC3350, TSC3395, TSC95 and TSCH).Razali et al., 2012 reported similarly that non-polar extract of Tamarind seed showed lower radical scavenging activity than polar extracts. This observation is fairly common in plants, as not many non-polar compounds are able to act as

potent antioxidant (Kulkarni et al., 2004 and Siddhuraju et al., 2007). However, Tsuda et al., 1994 reported that tamarind had markedactivity in the methanol extract and the ethylacetateextract, indicating that tamarind would have both polarand specific nonpolar anti-oxidative substances.

Table 2: Superoxide anion scavenging activity of Tamarind seed coat on various extractions*.

Samples	%inhibition				IC50 (µg/ml)
	1,000 µg/ml	500 µg/ml	50 µg/ml	5 µg/ml	
TSC331	82.3158	77.6842	52.2526	38.2739	13.9±0.04
TSC332	82.3579	79.4105	47.3263	34.8211	17.5±0.02
TSC3350	91.9575	87.7053	73.6421	61.2632	<5
TSC3395	100.4392	96.8765	76.0859	70.8638	<5
TSC50	90.4487	76.4103	61.0897	44.1026	11.1±0.09
TSC95	94.9856	93.5883	75.8323	54.9527	<5
TSCH	92.4407	90.6715	77.8448	72.4970	<5

* Results were expressed as the averages of triplicates ± S.D.

Mitochondrial dehydrogenase activity assay (WST assay)

To investigate of tamarind seed coat extracts on mitochondrial toxicity was measured in five cells line culture. The mitochondrial succinate dehydrogenase activity was representing for alteration of mitochondrial functions. The resulted show in concentration of extracts that inhibited succinate dehydrogenase activity for 50%. As shown in Table 3, cells were treated with Tamarind seed coat extracts for 24 h and the concentrations was showedranging from 15 to 1000 µg/ml inhibited enzyme activity. All of extracts occurred in the absence of any effect on enzyme activity except for TSCH extract. Caco2 cells were showed highest sensitivity effect on all of tamarind seed coat extracts. TSCH extract had strongly effect on cancer cells line,IC50 values were found to be 75.27, 15.06 and 100.37 µg/ml for HepG2, Hela and Caco2, respectively. Interestingly, IC50 values of TSCH extract were not effect on HK-2. Nakchat et al., 2014 has been reported that tamarind seed coat extract with water was not appearing on human foreskin fibroblast cells (CCD-10664sk). Moreover, Polysaccharide PST001 isolated from tamarind seed kernel was showed the anti-proliferation on some cancer cells (A549, KB and DLA) base on inhibition of mitochondrial dehydrogenase activity (Aravind *et al*., 2012).

Table 3: Mitochondrial dehydrogenase activity of various Tamarind seed coat extracts*.

IC50 (µg/ml)	Cancer cells			Normal cells	
	HepG2	Hela	Caco2	L929	HK-2
TSC331	>1000	>1000	920.59±103.52	>1000	> 1000
TSC332	>1000	>1000	873.26±83.54	>1000	> 1000
TSC3350	>1000	>1000	588.22±150.31	>1000	> 1000
TSC3395	> 1000	>1000	954.19±132.60	> 1000	> 1000
TSC341	>1000	>1000	343.77±102.88	>1000	> 1000
TSC342	>1000	>1000	654.46±163.37	>1000	> 1000
TSC50	>1000	>1000	602.50±130.25	>1000	> 1000
TSC95	>1000	>1000	249.21±49.7	>1000	> 1000
TSCH	75.27±10.54	15.06±2.54	100.73±11.84	444.16±45.56	> 1000

* Results were expressed as the averages of triplicates ± S.D.

Mitochondrial membrane potential (MMP assay)

Effect of mitochondrial membrane potential was importance in the detection of apoptosis. The mitochondrial membrane potential was detected with JC-1 staining. In healthy cell, JC-1 accumulates in the mitochondrial as JC-1 aggregates with intense red fluorescent and unhealthy cell or apoptosis JC-1 unable accumulates in the mitochondrial it shows only green fluorescent. The untreated Hela cell exhibited strongly aggregate red fluorescence slightly significant green (Figure 1). The degradation of mitochondrial membrane potential (green) showed strongly effect with Hela cells treated with valinomycin. HeLa cell treated with crude extracts from TSCH concentration 5, 15 µg/ml showed the results that ratio of green fluorescence more intense than red fluorescence. The intensity of JC-1 aggregate and monomer showed with arbitrary unit in Figure 2. The average ratio of mitochondrial JC-1 aggregate intensity (red) and JC-1monomer intensity (green) that showed in order as untreated cells (3.35)>TSCH 5 µg/ml (0.15)>TSCH 15 µg/ml (0.09)>valinomycin (0.06). Untreated cells (healthy cells) showed significant highest ratio of red/green. The ratio of red/green intensity of treated cells with valinomycin was similarly resulted with 15 µg/ml TSCH extracts.

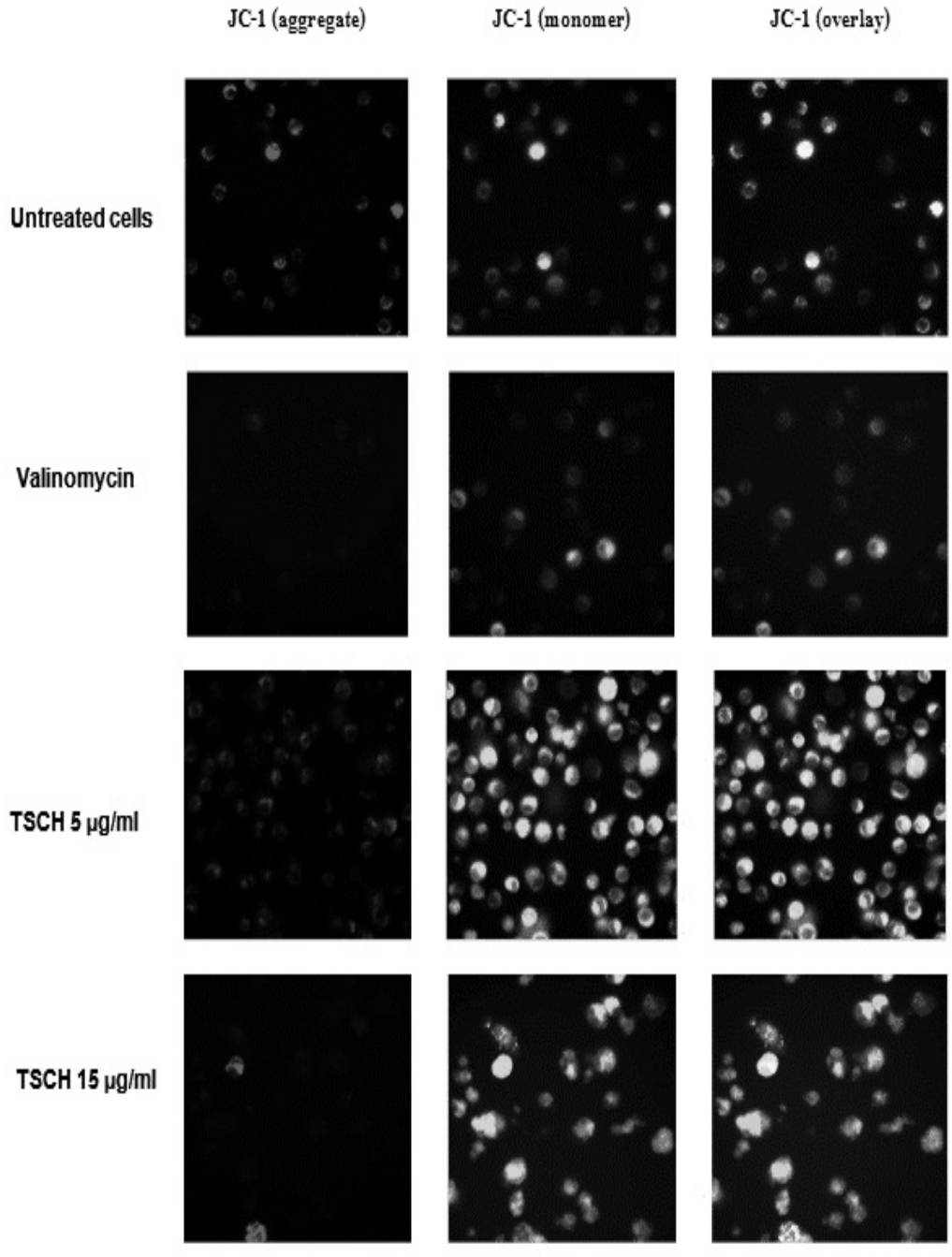

Figure 1: Fluorescence image of HeLa cells labeled with JC-1. Cells were treated with valinomycin 0.5 µg/ml and water extract of tamarind seed coat (TSCH) concentration at 5 and 15 µg/ml. Left and middle panels show the aggregate (red) and monomer (green) fluorescence, respectively. The right panels show the overlay of the two images; in this case the orange/yellow colour denotes co-localization of red and green fluorescence signals.

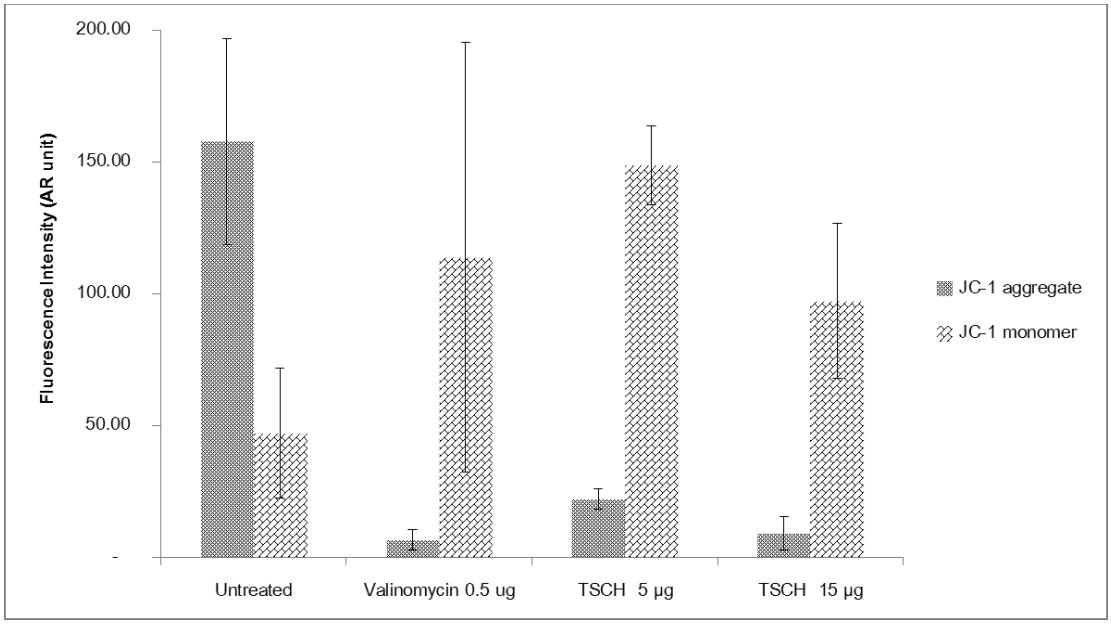

Figure 2: Effect of water extract of tamarind seed coat (TSCH) on JC-1 fluorescence intensity. Hela cells were incubate with TSCH at 5 and 15 15 µg/ml and compare with 0.5 µg/ml valinomycin (chemotheraputica agent). Fluorescence intensities were analyzed after 30 min JC-1 incubation. Values represent the mean±S.D. from ten fields of experiment.

4. Conclusion

Analyses of the free radical scavenging activity and cytotoxic on cancer cells of various extracts of tamarind seed coat, are useful in providing information on the potential of this plant as a source of anti-oxidant and anti-cancer. Overall, the water extract was most effective for extraction. From this study, it was found that the water extract of tamarind seed coat contained the most free radical scavenging activity and toxic with mitochondrial membrane of cancer cells. The high toxic effect of water extract on cancer cells implies the potential of this extract for use as an alternative compound of natural anti-cancer agent. In addi tional, this study also provides further scientific support for the medicinal use of this extract. The chemical analysis, identification and *in vivo* studies are required to further confirm.

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Reference

AravindSR, Joseph MM, Varghese S, Balaram P, et al. Antitumor and Immunopotentiating

Activity ofPolysaccharide PST001 Isolated from the Seed Kernel of *Tamarindus indica*: An In Vivo Study in Mice. *The Scientific World Journal 2012*; 361382: 1-14.

Bhattacharya S, Bal S, Mukherjee RK, Bhattach arya S. Functional and nutritional proper ties of tamarind (*Tamarindus indica*) kernel protein. Food Chem 1994; 49: 1-9.

De M, De Krishna A, Baneerjee AB. Antimicrobial screening of some Indian spices. Phytother Res 1999; 13(7): 616-618.

Kulkarni AP, Aradhya SM, Divakar S. Isolation and identification of a radical scavenging antioxidant–punicalagin from pith and carpellary membrane of pomegranate fruit. Food Chem 2004; 87: 551-557.

Luengthanaphol S, Mongkholkhajornsilp D, Douglas S, Douglas PL, et al. Extraction of antioxidants from sweet Thai tamarind seed coat—preliminary experiments. *J Food Eng 2004*; 63: 247-252.

Maiti R, Das UK, Ghosh D. Attenuation of hyper glycemia and hyperlipidemia in strepto zotocin induced diabetic rats by aqueous extract of seed of *Tamarindusindica*. Biol Pharm Bull 2005; 28: 1172-1176.

Maiti R, Jana D, Das UK, et al. Antidiabetic effect of aqueous extract of seed of *Tamarin dusindica* in strptozotocin-induced diabetic rats. *J Ethnopharmacol 2004*; 92: 85-91.

Nakchat O, Meksuriyen D, Pongsamart S. Anti oxidant and anti-lipid peroxidation activi ties of *Tamarindusindica* seed coat in human fibroblast cells.Indian *J Exp Biol 2014*; 52(2):125-32.

Nurhanani R, Sarni MJ, Amirah Fa, et al. Effects of various solvents on the extraction of antioxidant phenolics from the leaves, seeds, veins and skins of *Tamarindus indica* L. *Food Chem 2012*; 131 (2): 441-448.

Paula FS, Kabeya LM, Kanashiro A, et al. Modulation of human neutrophil oxidative metabolism and degranulation by extract of *Tamarindusindica* L. fruit pulp. *Food Chem Toxicol 2009*; 47: 163-170.

SiddhurajuP. Antioxidant activity of polyphenolic compounds extracted from defatted raw and dry heated *Tamarindusindica* seed coat. LWT 2007; 40: 982-990.

Sudjaroen Y, Haubner R, Wurtele G, *et al*. Isolation and structure elucidation of phenolic antioxidants from Tamarind (*Tamarindus indica* L.) seeds and pericarp. *Food Chem Toxicol 2005*; 43: 1673-1682.

TsudaT, Watanabe M, Ohshima K, et al. Antioxidative components isolated from the seed of Tamarind (*Tamarindus indica* L.). *J AGR Food Chem1994*; 42: 2671-2674.