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ORIGINAL ARTICLE

Evaluation of Cytotoxicity and Antioxidant Potential of Bael Leaf (*Aegle marmelos*) on Human Hepatocellular Carcinoma Cell Line

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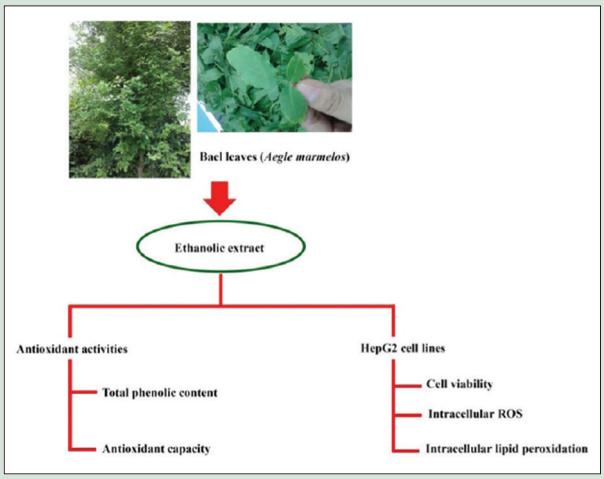
ABSTRACT

Background: *Aegle marmelos* (Bael) is one of the important medicinal plants for curing human diseases. Nevertheless, the information about the cytotoxic and antioxidant effects of Bael leaf is not much. **Objectives:** The objective of this study is to evaluate the cytotoxicity and antioxidant activity of Bael leaf in human hepatocellular carcinoma (HepG2) cell lines. **Materials and Methods:** Bael leaves extract was prepared by 80% (*v*/v) aqueous ethanol and measured for total phenolic compound and antioxidant capacity. The ability of ethanolic Bael leaf extract to inhibit hepatic cancer cells (HepG2 cell) growth was evaluated by determining the percentages of cell viability. The levels of intracellular reactive-oxygen species (ROS) and lipid peroxidation were determined. **Results:** Bael leaf extract demonstrated the potentiality to inhibit the cancer cell growth with a 50% inhibitory concentration (IC_{50}) at 50 µg/ml and 72 µg/ml after 24 h and 48 h incubated times, respectively. The total phenolic content and antioxidant capacity of ethanolic extract were 218.33 ± 43.81 mg gallic acid equivalent/g of dry extract and 33.62 ± 3.07 mg Trolox equivalent antioxidant capacity/g of dry extract, respectively. At the lowest concentration of Bael leaf extract, it was found that Bael leaf extract shows the antioxidant activity by the ability to lowering the level of ROS and malondialdehyde in HepG2 cell. **Conclusion:** Bael leaf has a high antioxidant component, which is beneficial and can be developed as new therapeutic uses. However, further studies on the benefits of Bael leaf should be performed for better realizing and effective use soon.

Key words: Antioxidant, bael, cytotoxicity, human hepatocellular carcinoma cell line, malondialdehyde, reactive-oxygen species

SUMMARY

- Bael leaf extract possesses potential cytotoxicity and antioxidant properties to the human hepatocellular carcinoma cell. Moreover, it was demonstrated that Bael leaf extract could decrease the levels of reactive-oxygen species in the cytosolic compartment of the human hepatocellular carcinoma cells. However, the study conducted here is preliminary.



Abbreviations Used: HepG2 cell: Human hepatocellular carcinoma cell lines; MDA: Malondialdehyde; ROS: Reactive oxygen species; IC_{50}: 50% inhibitory concentration; DCFH-DA: 2′;7′-dichlorodihydrofluorescein diacetate; DCFH: 2′,7′-dichlorofluorescein; DCF: dichlorofluorescein; FI: Fluorescence intensity; TBARS: Thiobarbituric acid reactive substance; MTT: 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; FBS: Fetal bovine serum; DMEM: Dulbecco's modified Eagle's medium; DMSO: Dimethyl sulfoxide; H_2O_2: Hydrogen peroxide; Trolox: 6-hydroxy-2,5,7,8-tetramethylchroman- 2-carboxylic acid; ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); BHT: Butylated hydroxytoluene; TCA: Trichloroacetic acid; H_3PO_4: Phosphoric acid; TBA: Thiobarbituric acid; GAE: Gallic acid equivalent; TEAC: Trolox equivalent antioxidant capacity; SEM: Standard error of the mean; ANOVA: One-way analysis of variance.

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INTRODUCTION

Aegle marmelos (L.) Correa (*A. marmelos*) is commonly known as Bael. It is a tree of the *Rutaceae* family, which has been widely used in the traditional systems of Indian medicinal plant due to its various medicinal properties. Bael grows in the tropical and subtropical regions of India and Southeast Asia, including Thailand and Myanmar.[1] Previous studies show the different parts of Bael, which include leaf, fruit, stem, and root, are used for the remedy of various diseases such as anemia, asthma, high blood pressure, fracture, jaundice, wound healing, and swollen joint.[2]

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Bael has been found to contain many bioactive compounds, including flavonoid, quercetin, cinnamic acid, cineole, aegeline, skimmianine, lupeol, citral, citronella, cuminaldehyde, marmin, marmesinin, eugenol, marmelosin, luvangetin, rutin, aurapten, tannin, psoralen, marmelide, and fagarine. Moreover, it has also been found that Bael leaves have hypoglycemic, antifungal, cardiotonic effect, antimicrobial and antiviral activities, anti-dyslipidemic, anti-cancer effect, analgesic, antioxidant activities, and anti-inflammatory activity.[1,3,4]

As many parts of Bael have medicinal values, some previous studies investigated the pharmacognostic standardization, evaluation of anti-proliferative activity, phytochemicals in the treatment and prevention of cancer of the leaf extracts *A. marmelos* (L.) Correa (*Rutaceae*) in various human cancer cell lines, including leukemia (THP-1), ovary (IGR-OV-1), colon (CoLo-205), lung (A-549), breast (MCF-7), and prostate (PC-3) cancer.[5,6] Nevertheless, few studies have been reported about the hepatoprotective effects of Bael leaf. Thus, the present study was to elucidate the cytotoxicity and antioxidant activity of ethanolic Bael leaf extract in human liver cancer cell lines.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS), Dimethyl sulfoxide (DMSO) (Fisher Scientific, UK), Folin-Ciocalteu's phenol reagent (Merck KGaA, Darmstadt, Germany), 0.5% trypsin-EDTA solution, hydrogen peroxide (H_2O_2) (35%), and penicillin-streptomycin were purchased from GIBCO® Invitrogen, CA, USA. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), BHT, Gallic acid, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), trichloroacetic acid, phosphoric acid, 1,1,3,3-tetramethoxypropane, and thiobarbituric acid (TBA) were bought from Sigma-Aldrich, St. Louis, MO, USA.

Plant materials and preparation of Bael leaf extract

Fresh Bael leaves were collected from Lampang, Thailand, during August 2014. The plant material was authenticated by Dr. Prachaya Srisanga, Herbarium Curator at the Botanic Garden Organization, Queen Sirikit Botanic Garden, Mae Rim, Chiang Mai, Thailand, and voucher specimens have been deposited in Queen Sirikit Botanic Garden, Chiang Mai, Thailand (Voucher K. Kulprachakarn-01). Bael leaves were immediately dried in an electric oven for 24 h at 60°C. The dried coarse powder of samples (100 g) was extracted in 1 L of 80% *(v*/v) aqueous ethanol overnight at the room temperature. After that, the crude extract was filtered through Whatman No. 1 filter paper, and the filtrate was concentrated by evaporating the solvent under vacuum on a rotary evaporator. The percentage yield of the extract was 27.20%. Before being used for further analysis, the dried residue was kept in the dark at 20°C.

Total phenolic content

The total phenolic content of the extracts was determined using the Folin-Ciocalteu method.[7] Bael leaf extract was mixed with 10% Folin-Ciocalteu's phenol reagent for 3 min, following by the addition of 7.5% *(w*/v) solution of sodium carbonate. The mixture was stirred and measured at 765 nm using a spectrophotometer after keeping in the dark for 30 min. Gallic acid was used as a standard. The results were expressed as mg of gallic acid equivalent (GAE)/g of dry extract, utilizing a calibration curve of gallic acid in a concentration range of 0–0.25 mg/mL.

Antioxidant capacity

ABTS radical cation-scavenging activity was analyzed according to method W Chen *et al*.[8] with minor variation. A stock $ABTS^{\bullet+}$ solution was freshly prepared by reacting of 2.45 mM potassium persulfate and seven mM of 2,2 azobis (2-amidinopropane) dihydrochloride in the dark for 12 h at the room temperature. The stock solution was then diluted with distilled water until an absorbance reached 0.70 ± 0.02 at 734 nm as the working solution. Determination of the antioxidant capacity, Bael leaf extract solution was added at a ratio of 1:100 *(v*/v) to $ABTS^{\bullet+}$ working solution, then incubated in the dark. After 6 min of incubation at room temperature, the optical density was read at 734 nm. A calibration curve was made by Trolox, and the results were expressed as mg of Trolox equivalent antioxidant capacity (TEAC)/g of dry extract.

Cell culture

Human hepatocellular carcinoma (HepG2) cell lines as an *in vitro* model for cytotoxicity studies and also have been widely used to study biochemical of a variety of chemicals and drugs.[9] The HepG2 cell was kindly provided by Professor Dr. Somdet Srichairatanakool, Department of Biochemistry, Faculty of Medicine, Chiang Mai University. The cells were cultured in complete DMEM medium supplemented with 100 IU/mL penicillin/streptomycin and 10% (*v*/v) of FBS in T-75 tissue culture flask. They were subsequently incubated at 37°C in a 5% CO_2 humid incubator.[10] When HepG2 cells reached 70%–80% confluence, cells were harvested. The cell suspension at a density of approximately 5×10^3 cells was plated in each well of 96 well plates. It was incubated at 37°C in a 5% CO_2 humid incubator overnight to allow attachment to the culture wells. Then, the medium was discarded, and the Bael leaf extract with different concentrations (6.25–200 μg/mL) was added to the well, and the incubation was continued for 24 h or 48 h. As a control, the HepG2 cells were cultured in the medium without the extract solution. All analysis was done in triplicate.

Cell viability assay

In vitro cytotoxic potential of Bael leaf extract against HepG2 cells was performed by the MTT assay.[11] After incubation, HepG2 cells with different concentrations of Bael leaf extract for 24 h, following by adding 10 μL (5 mg/mL) of MTT dye solution to each well for 4 h at 37°C. After the removal of the MTT dye solution, cells were treated with DMSO. The absorbance at 540/630 nm was quantified using a microplate spectrophotometer. The percentage of inhibition was calculated after comparing it with the control, and the cytotoxicity of the extract was expressed as the concentration of drug-inhibiting cell growth by 50% (IC_{50}) values.

Intracellular reactive-oxygen species

Intracellular production of reactive-oxygen species (ROS) was estimated by the oxidation-sensitive fluorescent probe; 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) assay.[12] DCFH-DA can simply diffuse into the cells and be hydrolyzed by esterase in viable cells to produce 2',7'-dichlorofluorescein, which will subsequently be oxidized by existing ROS to dichlorofluorescein (DCF). Cells were treated with a different concentration (0.3125–20 μg/mL) of Bael leaf extract for 24 h at 37°C, 5% CO_2. After that, the treated cells were washed with sterile PBS (three times) and labeled with ten μM DCFH-DA solution in the dark at 37°C, 5% CO_2 for 30 min. Afterward, cells were washed and were subsequently challenged with 125 μM H_2O_2 solution for 20 min in the dark. Then, the induction of cellular ROS production was immediately measured using a spectrofluorometer with $\lambda_{ex/em}$ 485/530 nm.[13] The fluorescence intensity (FI) of DCF

value, directly proportional to the amount of intracellular ROS and is responsible for the level of cellular oxidative stress.[14]

Intracellular lipid peroxidation

Malondialdehyde (MDA), a secondary product of lipid peroxidation, was adopted as an index of lipid peroxidation and was measured in intracellular by the TBA reactive substance (TBARS) assay.[15] Briefly, cells were treated with the test solutions as above at 37°C for 24 h. Afterward, cell pellets were collected by low-speed centrifugation and mixed with the lysis solution containing 10% (*w*/v) SDS and 0.4% (*w*/v) TBA in 10% acetic acid, pH 5.0. The total volume of the mixture was adjusted to 0.8 ml with distilled water and incubated at 90°C for 30 min. After cooling to room temperature, 0.8 mL *n*-Butanol was added to the lysate to extract the pink-colored MDA product. Then, the solution was shaken vigorously, centrifuged at 5000 rpm for 5 min, and the absorbance was then read at 532 nm using a spectrophotometer. Lastly, TBARS concentrations were calculated from a standard curve constructed by varying levels of 1,1,3,3-tetramethoxypropane (0–100 μM).

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM) from triplicate samples of three independent analyses. All statistical calculations were defined using a one-way analysis of variance of GraphPad Prism software (Version 7.00 for Windows, La Jolla, California, USA). P <0.05 was considered as statistically significant.

RESULTS

In the present study, Bael leaves were harvested from Lampang province, a Northern part of Thailand. The Bael leaf ethanolic fraction was further used to determine cytotoxicity potential, total phenolic content, and antioxidant properties.

Cytotoxic activity

The IC_{50} value refers to the concentration of the Bael leaf extract, which causes 50% mortality among HepG2 cells. As shown in Figure 1, the IC_{50} amount of the extract possessed significant bioactivity, and it was concentration-dependent. The IC_{50} amount of Bael leaf extract was found to be 50 and 72 μg/mL after 24 h and 48 h of incubation.

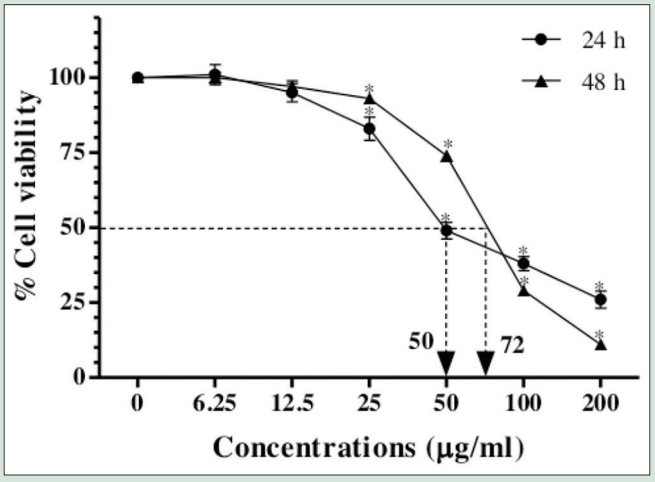

Figure 1: Cytotoxicity activity of Bael leaf extract at various concentrations (0–200 μg/mL) in human hepatocellular carcinoma cells for 24 h and 48 h of incubation. All results are presented as mean ± standard error of the mean of triplicate determinations. *P < 0.05 compared to non-treatment control

Estimation of total phenolic content and antioxidant capacity

The amount of total phenolic was assessed using Folin–Ciocalteu reagent. This method is commonly used for the quantification of the entire phenolic content in various plant extracts. The total amount of phenolic content in Bael leaf extract was 218.33 ± 43.81 mg GAE/g of the dry extract [Table 1].

In addition, the total antioxidant capacity of Bael leaf extract was measured by modified Trolox equivalent total antioxidant capacity (TEAC) assay using its ability to decolorized ABTS$^{\bullet+}$ radical cation.[16] As a result of Table 1, the antioxidant capacity levels of Bael leaf extract was approximately 33.62 ± 3.07 mg TEAC/g of dry extract.

Effect of bael leaf extract on intracellular reactive-oxygen species levels

As a result, shown in Figure 2, demonstrated that Bael leaf extract presented the antioxidant activity in the cultured HepG2 cells by

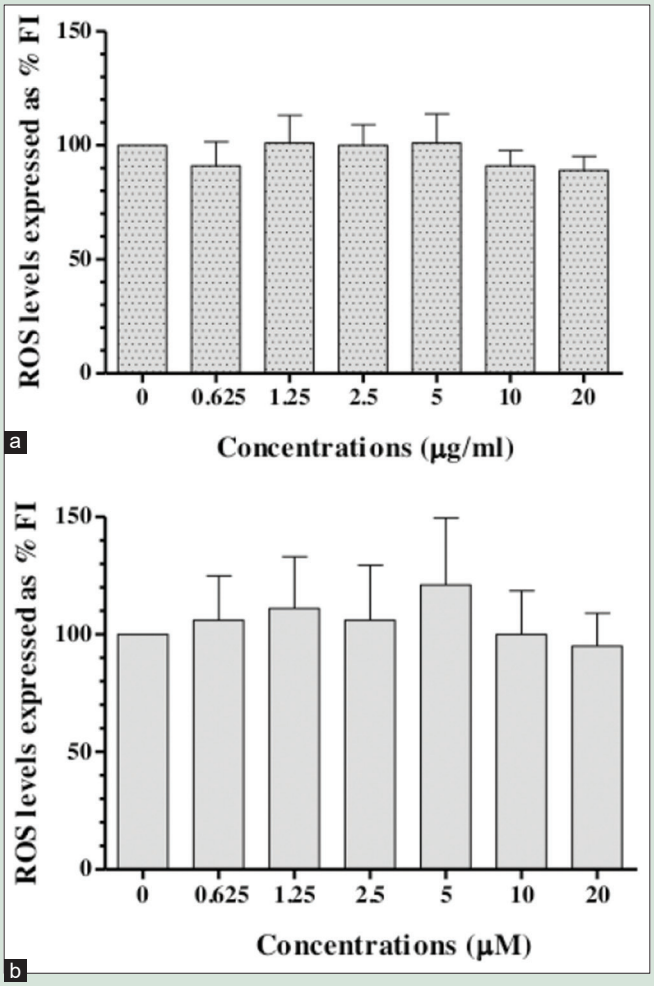

Figure 2: The reactive-oxygen species levels in 24 h-treated human hepatocellular carcinoma cells with Bael leaf extract (a) and trolox (b). Data are shown as mean ± standard error of the mean – three different experiments with three replicates (wells) each one (n = 3)

Table 1: Total phenolic content and antioxidant activity of Bael leaf extract

Extract	Total phenolic content (mg GAE/g of dry extract)	Antioxidant activity (mg TEAC/g of dry extract)
Bael leaf	218.33±43.81	33.62±3.07

Data are mean±SEM values of triplicate determinations. GAE: Gallic acid equivalent; TEAC: Trolox equivalent antioxidant capacity; SEM: Standard error of the mean

the reduction of cellular H_2O_2-derived-ROS levels. In this study, a comparable influence on ROS levels between Bael leaf extract [Figure 2a] and Trolox [Figure 2b] was observed for HepG2 cells. Notably, at a concentration of 20 μg/mL of Bael leaf extract highly diminished the ROS levels.

Effect of bael leaf extract on malondialdehyde concentrations

The amount of lipid-peroxidation product, MDA detected in Bael leaf extract-treated HepG2 cells [Figure 3a] was found to be slightly higher than Trolox-treated HepG2 cells [Figure 3b]. The ability of Bael leaf extract to inhibit H_2O_2-induced lipid peroxidation after the treatment with extract at a concentration of 1.25 μg/mL was highly reduced MDA production.

DISCUSSION

The cell viability of Bael leaf against the HepG2 cell line was determined by the MTT method. This method is based on the enzymatic cleavage of the soluble tetrazolium salt into a purple formazan by cellular mitochondrial dehydrogenases present in viable cells.

The previous studies reported the cytotoxicity activity of Bael leaf extracts against cell lines, including melanoma Colo38, leukemic K562, B-lymphoid Raji, T-lymphoid Jurkat, erythroleukemic HEL, breast cancer cell lines MCF7, and MDA-MB-231.[3] Marmelin, lupeol, eugenol, and citral, which are compounds present in *A. marmelos* demonstrated cytotoxic effects on various human cancer cell lines.[6,17,18] In addition, the experiment has also revealed that the LC_{50} values of three different Bael leaf extracts (methanol, ethyl acetate, and *n*-hexane) are 4.482 μg/mL, 5.278 μg/mL, and 5.278 μg/mL indicating the presence of the potential cytotoxic property.[19]

Anshu Wali *et al*.[20] reported the amount of total phenolic of *A. marmelos* leaf methanol extract and ethyl acetate fraction ranged from 16.5 ± 0.3–23.2 ± mg GAE/g dry weight, respectively. Polyphenols, particularly flavonoids, which are hydroxyls containing secondary metabolites present in plants and exhibit free radical-scavenging activities.[21] It has been used to treat many human diseases, such as cancer, coronary heart diseases, and diabetes.

As the above results, it may be summarized that the presence of plenty of phenolic compounds in Bael leaf extracts promoted to its strong antioxidant potential. The antioxidant effect of Beal leaf extract is assumed to the free radical-scavenging property due to the presence of flavonoids and phenolics.[22]

The antioxidant activity found in Bael leaf extract impels us to estimate a possible protective effect of the extracts against ROS generated in the HepG2 cells. The oxidative stress response is modulated by an imbalance between the production of ROS and the capability of an antioxidant enzyme to eliminate ROS.[23] It is caused by elevated intracellular levels of ROS. Antioxidants can prevent oxidative damage induced by ROS by the prevention of ROS construction, disruption of ROS attack, and scavenging-reactive metabolites. The results have shown that the natural antioxidants of Bael leaf extract sharply decrease the ROS levels in the HepG2 cell,

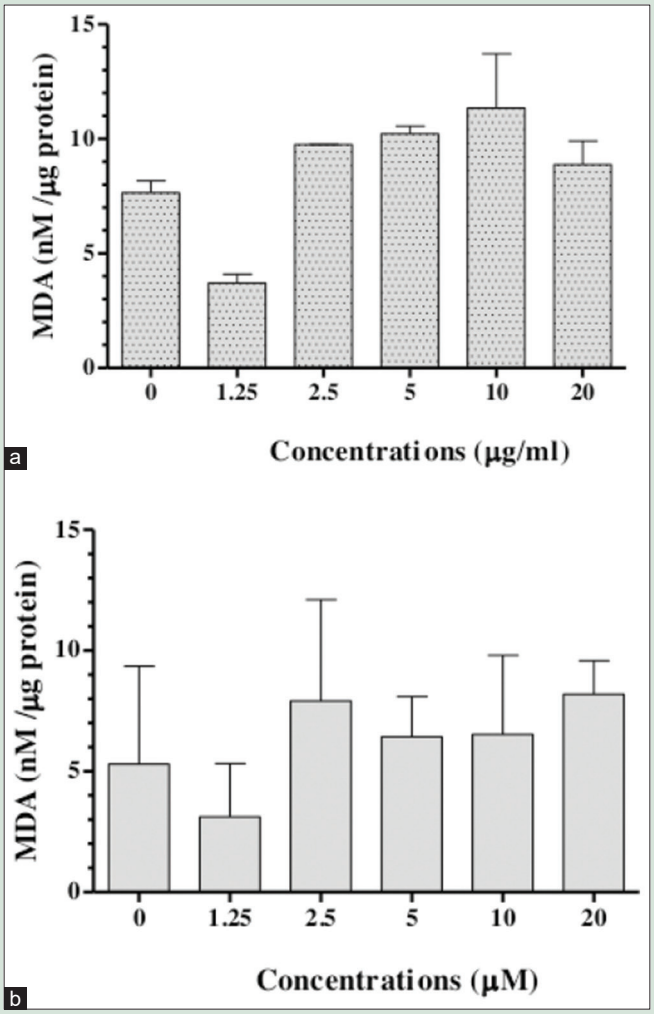

Figure 3: The levels of malondialdehyde in 24 h-treated human hepatocellular carcinoma cells with Bael leaf extract (a) and Trolox (b). Malondialdehyde concentrations were determined by using the thiobarbituric acid reactive substance method. Data are shown as mean ± standard error of the mean – three different experiments with three replicates (wells) each one ($n = 3$)

thus may prevent or inhibit conditions that promote oxidative stress in the cell.[24]

MC Sabu and R Kuttan[25] revealed that a methanolic *A. marmelos* leaves extract to show the effective reduction of blood sugar and against the oxidative stress by decreasing lipid peroxidation levels in alloxan-induced diabetic rats. Furthermore, the antioxidant enzyme levels, which are superoxide dismutase, catalase, glutathione, and glutathione peroxidase, were found to be increased after drug administration. The leaf extracts also prevented the radiation-induced lipid peroxidation in the intestine, spleen, kidney, and liver of mice with a concomitant increase in the levels of glutathione.[6] The extractions of Bael leaf in water, ethanol, and methanol, are capable of preventing the MDA formation of TBARS generated by Fenton's reagent.[26]

CONCLUSION

In conclusion, the present study indicated that Bael leaf extract possesses potential cytotoxicity and antioxidant properties to the HepG2 cell. Moreover, it was demonstrated that Bael leaf extract could decrease the levels of ROS in the cytosolic compartment of the HepG2 cells.

However, the study conducted here is preliminary. Further studies are needed to carry on and clarify these findings for better realizing the pharmacological activities, mechanism of action, and the active compounds for these actions.

Acknowledgements

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Financial support and sponsorship

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Conflicts of interest

There are no conflicts of interest.

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