

ANTIPROLIFERATIVE, ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC CONTENT OF *MITRAGYNA PARVIFOLIA* (ROXB.) KORTH

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ABSTRACT

Objective: In the present study bark and leaves of *Mitragynaparvifolia*(Roxb.) Korth were evaluated for total phenolic content, total flavonoid content, antioxidant potential, lipid peroxidation and antiproliferative effect on HeLa cell lines.

Methods: Dried bark and leaves were suspended in five different solvent systems viz; distilled water, methanol, acetone, ethyl acetate and hexane. Total phenolic content was estimated using Folin-Ciocalteu reagent and phenolic profiling by using high performance liquid chromatography (HPLC). Antioxidant potential and flavonoids estimation were investigated using DPPH radical scavenging activity and aluminium chloride method respectively. Further lipid peroxidation and antiproliferative effect were observed using TBARS and MTT assay, followed by cell morphology using Giemsa and Acridine orange staining.

Results: The acetone extract of bark (57.08 ± 6.16 mg/mL) and leaf (60.1 ± 3.74 mg/mL) exhibited the highest phenolic content. Distilled water extracts of bark ($94 \pm 0.05\%$) and leaf ($95.63 \pm 0.34\%$) showed maximum antioxidant potential. Percent protection was observed maximum in acetone extracts of bark ($19.04 \pm 0.02\%$) and leaf ($25.11 \pm 0.09\%$). Total flavonoid content was found maximum in distilled water leaf extracts (0.869 ± 0.001 mg/mL). Acetone extracts showed high cytotoxic effect on HeLa cells compared to other extracts with very minimal or no cytotoxicity contributed by the vehicle (acetone) itself in comparison to positive control of 5% ricin.

Conclusion: Acetone extracts exhibited highest cytotoxic effect on HeLa cells compared to other extracts. These results also indicated that acetone extract have highest phenolic content and percent protection in lipid peroxidation. Thus *Mitragynaparvifolia*'s acetone extracts can attribute to one of the several bioactive compounds which can be further studied in detail.

Keywords: *Mitragynaparvifolia*, Antiproliferative activity, Antioxidant activity, MTT assay, HPLC, Flavonoids, Total phenolic content.

INTRODUCTION

Mitragynaparvifolia (Roxb.) Korth belongs to the Rubiaceae family and is widely used in ayurveda system of medicine as well as in Maratha local folklore. It is a deciduous, perennial tree that grows up to a height of 45 m in deep drained soil all over the Indian subcontinent, commonly referred to as Kaim, Kadamb or Neerkadambu. Commercially, it is widely grown in the Indian Thar desert. The bark and root are used in folk medicine for treatment of fever, colic, muscular pain, burning, gynaecological disorders, poisoning, edema and cough [1]. The bark is commercially used in timber industry for construction [2] as well as in paper industry [3]. It also exhibits anthelmintic [4], antimicrobial [5], and anxiolytic [6] activities. Leaves of *Mitragynaparvifolia* are also known for its analgesic [5], anti-inflammatory [7], anti-nociceptive [8], anti-convulsant [9], antipyretic, and antiarthritic [10]. They are also used to alleviate pain and swelling in wounds [4]. Analgesic and anti-inflammatory activities are also observed in the fruit of the plant [7], fruit juice can also be used as a lactodepurant [4]. *Mitragynaparvifolia* is a good source of fuel wood [11].

Mitraphylline, Isomitraphylline, Pteropodine, Isopteropodine, Speciophylline, Uncarine are the major oxindolic alkaloids isolated from *Mitragynaparvifolia* [3]. Rotundifoline, Rhynchophylline, Isorotundifoline, Rhynchociline, Speciociliatine, Speciofoline, Mitragynine are some other alkaloids found in the plant. However, the alkaloidal content in a plant varies with the geographical location and environmental conditions. Apart from alkaloids, other compounds like pyroligneous acids, aldehydes, ketones, scopoletin, thermophyllin, daucosterol, quinovic acid, β -sitosterol and methyl acetate are found in the plant [3].

Mitragynaspeciosa was known to be used as an opium substitute to heal opium addiction in the 19th century. *Mitragynainermis* is used to treat several diseases such as fever, headache diarrhoea, dysentery, cholera,

malaria and other diseases in folk medicine [12]. It is also attributed with antiplasmodial activity [13], antibacterial activity [14] and cardiovascular properties [15]. Further it is also known to exhibit cytotoxicity, genotoxicity, as well as acute and chronic toxicity [16].

Mitragynaparvifolia has been studied for its innumerable medicinal properties but bark and leaves of the plant are yet not explored for antioxidant and antiproliferative activity. Therefore in the present investigation attempt has been made to evaluate antioxidant and antiproliferative activities of *Mitragynaparvifolia* bark and leaves in different solvent systems including distilled water, methanol, acetone, ethyl acetate and hexane.

MATERIALS AND METHODS

Plant material

Bark and leaves of *Mitragynaparvifolia* were collected from Bamandongri region ($18^{\circ}58' 16''N$, $73^{\circ}1'27''E$) of Ulwe node, CBD Belapur, Navi Mumbai, Maharashtra during the month of December. The collected plant parts were air dried in a well-ventilated room at $37^{\circ}C$ temperature.

Extraction of sample

Dried bark and leaves of the plant were crushed into fine powder using liquid nitrogen. Ten grams of bark and leaf sample were suspended in 60 mL of different solvent systems viz; distilled water, methanol, acetone, ethyl acetate and hexane for overnight extraction. Extracts were then filtered through muslin cloth and concentrated using rotary evaporator at $56^{\circ}C$ [17].

Total phenolic content

Total phenol content (TPC) of the plant extracts was estimated using Folin-Ciocalteu reagent [17]. The extracts were mixed with Folin-

Cioalteau reagent and sodium carbonate (20%); the mixtures were incubated at room temperature for 30 min. The colorimetric change was observed at 650nm using a spectrophotometer. Total phenolic values were expressed(mg/mL) in terms of tannic acid equivalents as standard.

DPPH radical scavenging activity

The ability to scavenge free radicals was estimated using DPPH radical scavenging activity [18]. A 0.3mM solution of DPPH in methanol was prepared, of which 0.5mL of this solution was mixed with 100µL of bark and leaf extracts. The mixture was kept in dark at 37°C for 30 min. The absorbance was measured spectrophotometrically at 517nm. The ability to scavenge DPPH radical was calculated using following formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of the control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Lipid peroxidation assay

Mitochondrial extracts were obtained from Wistar rats (weighing about 240±20 g and 3 months old) [17]. Protein estimation of mitochondrial fraction was carried out by Bradford Reagent. Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 mL 95% ethanol in presence of 100 mL 85% (w/v) phosphoric acid to prepare Bradford reagent [19]. Once the dye was dissolved completely in solvent, the final reagent was diluted to 1L and filtered using Whatman No.1 paper before use. Protein was estimated spectrophotometrically at 595nm and mitochondrial pellet was suspended in potassium phosphate buffer at concentration of 10mg protein/mL.

Ascorbate-Fe²⁺-system is used to induce oxidative damage in mitochondrial fraction [19]. Malondialdehyde or MDA (TBARS) readily forms adduct with TBA reagent which can be measured spectrophotometrically by its characteristic pink color at 532nm after accounting for the appropriate blanks. Malondialdehyde standard was prepared by the acid hydrolysis of tetraetoxypropane[18].

Total flavonoids content

The flavonoids content of the extracts was estimated using Aluminium chloride reagent. The extracts (0.5mL) were mixed in equal volumes with 2 % Aluminium chloride solution in methanol (0.5mL) and were incubated for 10 min at room temperature. Absorbance was spectrophotometrically recorded at 368nm. The flavonoids content was expressed in Quercetin mg /mLequivalents[18].

HPLC characterization

Phenolic compounds were estimated on a Waters RP-HPLC (Model 2487), using a hypersil C18 reversed phase 15cm with 5µ particle size. A constant rate of 0.75mL/min was used with two mobile phases viz, phase A (1% acetic acid+ 20% methanol in water) and phase B (1% acetic acid+ 80% methanol in water). The elution gradient was started over 60 min, using an UV detector set at wavelength 280 nm. Identification of phenolic compounds from each sample was carried out by comparing their relative retention time with the standards of mixture chromatogram. The standards were used: catechol, vanilline, gallic acid, ferulic acid, p-coumaric acid and caffeic acid. The concentration of an individual compound was calculated on the basis of peak area measurements and converted to ppm. All chemicals and solvents used were HPLC spectral grade[19].

MTT cytotoxicity assay

Cytotoxicity assay using MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was performed as per[20]. HeLa cells were plated on a 96 well plate at a density of 2000 cells/well and incubated for 24 h at 37°C in 5% CO₂ incubator. The cells were then treated with a series of dilutions of the extract in 10% DMEM followed by incubation (5% CO₂) was carried out at 37°C for 24 h [21]. Negative control cells were treated with 10% DMEM (Dulbecco's Modified Eagle's medium). Vehicle control cells were treated with respective solvent systems devoid of extract. Positive control cells were exposed to 5% ricin. The added dilutions of the extract were then removed and cells were washed with saline to remove precipitation. Fresh 10% DMEM was then added to all wells.

After 24h, MTT dye (5mg/mL) diluted in fresh 10% DMEM was added to each well and incubated for 4 h. During incubation, the mitochondrial enzymes present in viable cells (succinate

dehydrogenase) cause reduction of MTT into insoluble purple formazan product. The formazan product was dissolved in crude DMSO and amount was estimated by measuring the absorbance at 545nm using ELISA plate reader wherein crude DMSO was used as blank[22]. The extracts were also tested by same protocol on bone marrow derived mesenchymal stem cells for their cytotoxicity.

$$\% \text{ Cell viability} = \frac{\text{OD sample}}{\text{OD negative control}} \times 100$$

Cell viability assay (in situ dye exclusion using trypan blue)

Cell viability assay was performed in situ using trypan blue[20]. Cells were seeded in a 96 well plate to which sample concentrations were added alongside a negative, positive and vehicle control. After 24 h exposure to the plant extract, cells were washed using saline. Ten µL of trypan blue was added to each well and cell count was performed using microscope after an exposure of 1min. Excessive dye was removed.

The following formulae were used for determining % cell death and % cell viability:

$$\text{Total count} = \text{average count} \times 2 (\text{dilution factor}) \times 0.5 \times 10^4$$

$$\text{Cell viability: \% viability} = \frac{\text{Total live cells}}{\text{Total no of live \& dead cells}} \times 100$$

$$\text{Cell death: \% death} = 100 - (\% \text{ viability})$$

Determination of apoptosis by staining procedures

Giemsa staining

Giemsa's solution is a mixture of methylene blue, eosin, and azure B. Each apoptotic body can be easily differentiated from a normal cell under a light microscope [23] due to which Giemsa staining was performed as a confirmatory test for apoptosis being present. After 24 h exposure to extracts, cells were washed with saline and stained with Giemsa for 1min. The excessive stain was removed by saline wash and observed under light microscope.

Acridine orange staining

Acridine orange a nucleic acid selective, fluorescent, cationic dye was useful for cell cycle determination. It is commonly used for the determination of apoptosis in cells as changes within the nucleus; cellular shrinkage etc could be clearly discerned. HeLa cells were plated in 35mm culture dishes at a density of 0.25 X 10⁶ cells and incubated for 24hr at 37°C in 5% CO₂ incubator. After 24 h exposure of grown cells in the extract, they were washed with phosphate buffer saline. The cells were then fixed using paraformaldehyde solution and dehydrated using methanol. The fixed cells were then stained using acridine orange (5µg/mL) and observed under fluorescence microscope [24].

Statistical Analysis

All the observations were taken in triplicate and the data was presented as ± standard deviation (SD). Analysis of Variance (one way ANOVA) was further performed using SPSS software (version 14.0). Correlation coefficient was calculated using Pearson Moment Correlation. A probability of p<0.001 was considered to be statistically significant and correlation coefficient @ values were determined [19].

RESULTS

Total phenolic content

Acetone extracts were found to have the highest phenolic content while least phenolic content was observed in hexane extracts (Table 1). Acetone leaf extract exhibited the highest total phenolic content (60.1±3.74mg/mL) followed by acetone bark extract (57.08±6.16 mg/mL). The phenolic content in the present study decreases in the following order of solvents: acetone > methanol > distilled water > ethyl acetate > hexane.

DPPH radical scavenging activity

Distilled water extract of leaf (95.63±0.34%) showed the greatest radical scavenging activity whereas hexane bark extract showed the

least (43.13±0.87%) (Table 1). The DPPH radical scavenging activities of the samples in the present study decreases in the

following order of solvents: distilled water > ethyl acetate > methanol > acetone > hexane.

Table 1: Estimation of total phenolic content, DPPH radical scavenging activity, total flavonoids and percent protection in lipid peroxidation of bark and leaf extracts of *M. parvifolia* plant

Parameters Studied	Plant parts	Solvents Used				
		Distilled Water	Methanol	Acetone	Ethyl acetate	Hexane
Total Phenol content (mg/mL)	Bark	47.27±3.339	54.5±6.95	57.08±6.16	27.14±0.70	2.19±0.63
	Leaf	50.37±6.13	51.09±1.11	60.1±3.74	40.05±0.13	11.94±1.23
DPPH radical scavenging activity (%)	Bark	94±0.05	92.09±0.23	88.61±0.45	27.62±0.75	43.13±0.87
	Leaf	95.63±0.34	90.06±0.89	51.92±0.78	47.33±0.65	46.48±0.54
Total flavonoids (mg/mL)	Bark	0.484±0.002	0.148±0.003	0.134±0.007	0.238±0.004	0.276±0.004
	Leaf	0.869±0.001	1.59±0.03	0.831±0.002	0.376±0.005	0.457±0.006
Percent protection (Lipid peroxidation)	Bark	1.54±0.04	13.54±0.01	19.04±0.02	5.35±0.07	3.25±0.04
	Leaf	1.34±0.06	14.6±0.02	25.11±0.09	6.99±0.05	4.23±0.22

Lipid peroxidation assay

Inhibitory effect of the plant extract on lipid peroxidation in rat liver mitochondria has been observed in (Table 1). Among all solvent systems, the greatest percent protection was found in acetone extracts of leaf and bark with a percent protection of 25.11% ± 0.09 and 19.04% ± 0.02 respectively.

Total Flavonoid Content

Highest total flavonoid content was observed in distilled water extracts of leaf (0.869±0.001mg/mL) and lowest in acetone extract of bark (0.134±0.007mg/mL). However, overall methanol seems to be a

good solvent for extraction of flavonoids especially from the leaf of *Mitragynaparvifolia* (Table 1).

HPLC analysis for polyphenol characterization

Polyphenolic compounds such as gallic acid, caffeic acid, ferulic acid, p-coumaric acid, vanillin and catechol were detected using RP-HPLC. Detection of each of these standards was observed in individual extracts (Table 2). Gallic acid (94.608 ppm), catechol (1669.51 ppm) and vanilline (477.451 ppm) was found highest in acetone bark extracts. Bark extract of acetone exhibited highest total phenolic content in correlation with data from total phenol content estimation using Folin -Ciocalteu assay.

Table 2: HPLC characterization of polyphenols present in bark and leaf extracts of *Mitragynaparvifolia*

Solvent Used	Plant parts	Gallic acid (ppm)	Catechol (ppm)	Caffeic acid (ppm)	Vanilline (ppm)	p-Coumaric acid (ppm)	Ferulic acid (ppm)
Distilled water	Bark	1.583	3.603	1.821	-	0.404	0.229
	Leaf	3.831	-	0.007	0.027	0.012	0.010
Methanol	Bark	1.638	2.335	15.783	-	9.668	-
	Leaf	0.038	0.022	95.483	-	-	4.792
Acetone	Bark	94.608	1669.51	-	477.451	0.814	0.984
	Leaf	-	45.58	21.359	128.554	60.196	7.946
Ethyl acetate	Bark	1.234	-	-	12.663	-	9.673
	Leaf	12.139	44.238	-	-	16.78	-
Hexane	Bark	-	-	-	7.883	13.12	-
	Leaf	0.896	23.23	15.67	-	-	19.64

Cytotoxicity assay - MTT assay

The cytotoxicity of the extracts was observed using the MTT assay on HeLa cell line. Four different extracts were selected for testing cytotoxicity on HeLa cells as follows viz; acetone bark and leaf since they displayed highest phenolic content; distilled water bark and leaf showed highest antioxidant potential. It was

observed that the acetone leaf extracts exhibited the maximum cytotoxicity on HeLa cell lines (Table 3). 5% of the extract was required to bring about 100% cell deaths (IC₁₀₀). Distilled water leaf extracts had the minimum activity on HeLa cells (Table 3). Extracts were tested on bone marrow mesenchymal cells (Table 3) and was found to be non-cytotoxic indicating specificity towards the HeLa cells.

Table 3: MTT cytotoxic assay results of acetone leaf extract

	Concentrations of Acetone leaf extract (Human bone marrow mesenchymal cells)							
	10%	5%	2.5%	1.25%	0.62%	0.31%	0.15%	0.07%
% Cell viability	99.46±0.2	99.19±0.6	98.65±0.3	98.92±0.2	98.92±0.1	99.46±0.2	99.73±0.6	99.19±0.3
% Cell death	0.54±0.4	0.81±0.9	1.34±0.5	1.08±0.7	1.08±0.8	0.54±0.3	0.269±0.03	0.81±0.23
	Concentrations of Acetone leaf extract (HeLa cells)							
	5%	2.5%	1.25%	0.62%	0.325%	0.12%	0.08%	
% Cell Viability	8.74±0.22	15.73±0.36	18.40±0.5	19.29±0.3	35.72±0.23	78.20±0.1	88.16±0.12	
% Cell Death	91.26±0.5	84.27±0.3	81.60±0.29	80.71±0.33	64.28±0.7	21.80±0.34	11.84±0.39	

In-situ dye exclusion (trypan blue)

Determination of cell viability and cell death was performed by *in-situ* dye exclusion test. The results obtained are in positive correlation with the cytotoxicity data obtained in MTT assays. Trypan blue assay provided a strong evidence for potential antiproliferative property of *Mitragynaparvifolia* extracts as observed in HeLa cells (Figure 1).

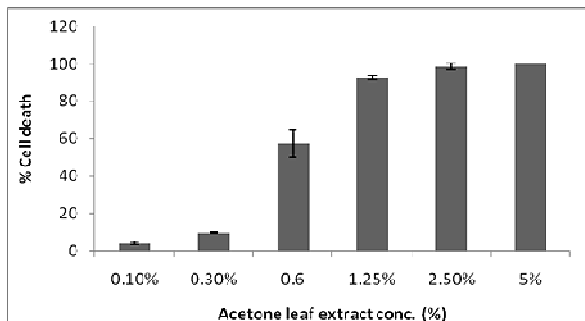


Fig. 1: *In situ* dye exclusion assay (trypan blue) for acetone leaf extract

Determination of cell apoptosis by staining

Giemsa

Apoptotic bodies were clearly distinguished from viable cells using giemsa stain (Figure 2a and Figure 2b). Cell apoptosis was visualized as shrunken with condensed and fragmented nuclear matter and sparse cytoplasm, whereas healthy cells were large, flattened with lightly stained abundant cytoplasm and darkly stained intact nucleus under light microscope.

Acridine orange

Healthy viable cells were visualized with well rounded, large and intact green nuclei (Figure 3a) while the cells underwent apoptosis display shrunken nuclei with minimal amount of cytoplasm as compared to the healthy cells (Figure 3b). In case of acetone extract of *Mitragynaparvifolia* leaf exhibited maximum amount of nuclear damage as compared to control, indicating cells undergoing cell death by apoptosis. Similarly, minimum nuclear damage was observed for distilled water extract of leaf in agreement with the MTT cytotoxicity and *in situ* dye exclusion tests.

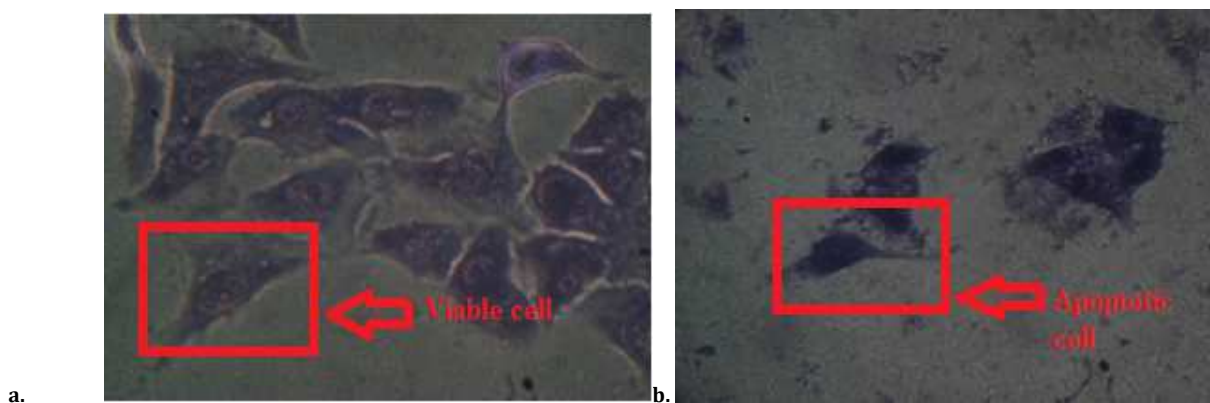


Fig. 2: a. Giemsa staining in control HeLa cells.
b. Giemsa staining in acetone bark extract treated HeLa cells.

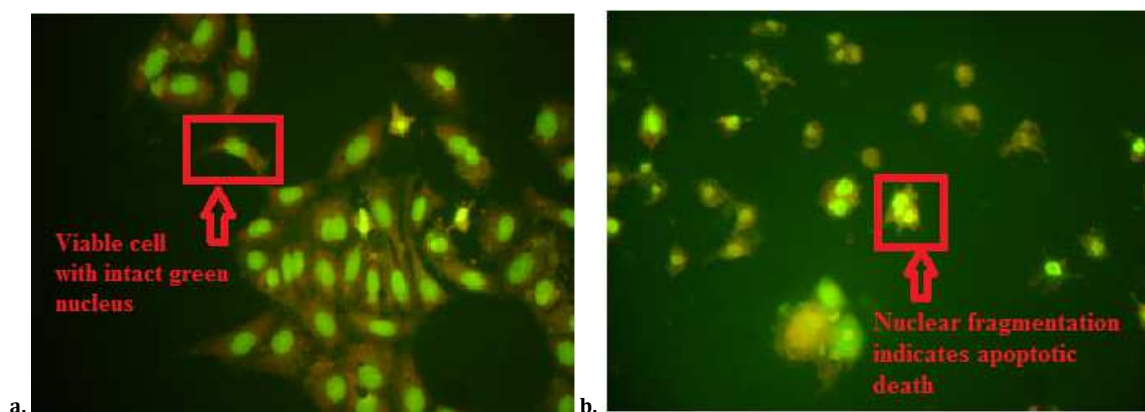


Fig. 3: a. Acridine orange staining in control HeLa cells (20X).
b. Acridine orange staining for D/W leaf extracts showing low discernable apoptotic damage (20X).

Correlation analysis between phenolic content, flavonoid content, antioxidant activity and lipid peroxidation

A correlation analysis was performed using Pearson correlation coefficient to check linear correlation among the assays. It was observed that there is a positive correlation between total phenol

content with DPPH antioxidant capacity ($r=0.57$, $p<0.001$) as well as weak positive correlation with lipid peroxidation ($r=0.03$, $p<0.001$). Interestingly we observed negative correlation between total phenol content and total flavonoid content ($r=-0.027$) and there was also negative correlation between total flavonoid and lipid peroxidation ($r=-0.04$, $p<0.001$) (Table 4).

Table 4: Correlation between total phenolic content, antioxidant activity and lipid peroxidation

Comparison n=10	TPC	TFC	DPPH	LP
TPC	1	-0.27	0.57*	0.03*
TFC	-	1	0.03*	-0.04*
DPPH	-	-	1	0.17
LP	-	-	-	1

* indicates significance at $P < 0.001$ TPC = Total phenol content TFC = Total flavonoid content LP = Lipid peroxidation n = number of samples (Considering extract of bark and leaves)

DISCUSSION

Polyphenols are of complex nature, they may be linked to the plant cell walls by sugars [25]. Acetone is a less polar solvent compared to methanol, distilled water and ethanol, and therefore is more efficient in cell wall degradation having nonpolar character which allows anthocyanins and other such polyphenols to be released [26]. Acetone is an effective solvent for extraction of condensed tannins which are relatively high molecular weight compounds, flavonols, and other high-tannin plant material as observed in study of lentil seeds [27]. Acetone extracts exhibiting the highest phenolic content can be indicative for the presence of flavonols or condensed tannins in *Mitragynaparvifolia* extracts. Plants produce various antioxidant compounds to combat reactive oxygen species posing an oxidative stress [28]. [29] stated that it is not necessary to correlate antioxidant activity with the high amounts of phenols. Antioxidant activity is strongly dependent on the solvent due to the different antioxidant potentials of phytochemical compounds with distinct polarities and extractability [30]. The low DPPH scavenging activity of acetone extracts could be resulting due to absence of appropriate scavenging compound against DPPH, despite exhibiting a good total phenolic content. Antioxidant properties of single compounds within a group can vary remarkably, so that the same levels of phenolics do not necessarily correspond to the same antioxidant responses [31]. High antioxidant activity in the methanolic extract of leaves of *Mitragynaparvifolia* was compared to chloroform extract [32]. Lipid peroxidation is caused due to reactive oxygen species (ROS) which is responsible for the deterioration of food by leading the formation of potential toxic compounds [33]. The concentration of peroxide decreases with the increase in the antioxidant activity [34], while the absorbance values are much smaller with higher antioxidant activities of the samples. The TBA assay is not specific for malondialdehyde (MDA) which is one of the breakdown products of lipid peroxidation. The non-specificity probably results from the acid eating step of the TBA assay that causes the formation of artificial TBA/MDA-like derivatives [35]. The DPPH scavenging activity was found to be in agreement with the % protection activity of the extracts of *Mitragynaparvifolia*. Phytochemicals such as phenolics, anthocyanins and other flavonoids contribute antioxidant activities in plants [36]. Ethanolic and methanolic extracts were found to be non-toxic when fed to mice for other studies at various concentrations of 5000 mg/kg of body weight [1], proving that extracts prove to be non-toxic to normal cells. Correlation analysis clearly determines that assay such as total phenol content, DPPH radical scavenging activity and lipid peroxidation correlates with each other. But total flavonoid content has negative correlation with total phenols and lipid peroxidation. Similar kind of correlation analysis was performed by using Pearson correlation coefficient [37, 19].

CONCLUSION

It is concluded from the study that acetone extract shows the highest phenolic content and highest anti-proliferative activity but relatively low antioxidant potential. Thus, the anti-proliferative properties could be attributed to the phenolic content. The microscopic examination exhibited considerable, visually evident apoptotic damage in HeLa cell population following exposure to crude extracts of *Mitragynaparvifolia*. Hence, such finding indicates that isolation and purification of the bioactive compounds could be necessary to explore the potent anti-proliferative potential of the plant.

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