RESEARCH ARTICLE

In vitro antioxidant and anticancer activity of *Blumea lacera* leaf extract

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The melanoma is a form of skin cancer that is caused by excessive exposure to ultraviolet radiation. The available chemotherapy does not show encouraging effect and therefore a new direction in the discovery of therapeutics is the need of the hour. Plant-based therapeutics has been a rich area of investigation in the field of drug discovery. In this paper, the antioxidant, and anticancer properties of the crude leaf extract from *Blumea lacera* were studied. Methanol and ethanol-based extracts were analyzed by using 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity, ferric reducing antioxidant power assay, thiobarbituric acid reactive substances for evaluation of antioxidant activity, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, wound healing assay, and colony formation assay were used to assess the anticancer activity of these extracts. The polyphenol content of the extracts was evaluated using the total phenolic content assay and high-performance liquid chromatography analysis. Results showed that the leaf extracts had antioxidant and anticancer activity. The anti-migratory effect of the methanolic extract against the melanoma cell line was also shown. This study has a definite value for future research and utilization of these plant extracts in anticancer drug development.

Keywords: *Blumea lacera*; antioxidant; anticancer.

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Introduction

Blumea lacera (*Burm*. *f*.) DC is a commonly found weed of family *Asteraceae*, which widely distributed in India. It is also found in many other countries such as Ceylon, China, Malaysia, Australia, and countries in tropical Africa [1]. In Asian countries like India, medicine practitioners have been using *Blumea lacera* in homoeopathic systems of medicines [2-3]. Different parts of the plant possess valuable medicinal properties [4-6]. The plant is known for containing phenolics, flavonoids, and essential oils [7]. The plant has a strong odor of turpentine and is used as an anti-helminthic and diuretic in indigenous medicine [8]. The plant is known for its several biological activities like astringent, stimulant, anthelmintic, antimicrobial, anti-inflammatory, and diuretic [9]. It exhibits a suppressive effect over the replication of HSV-1 and HSV-2 [10]. *Blumea lacera* is used in traditional medicine as an anticancer drug in combination with *Adhatoda vasica* and *Achyranthus aspera* [11]. *B. lacera* demonstrates moderate to mild antileukemic activity against L1210, P3HR1, anti-K562, Raji, and U937 leukemia cells [10].

Other species from the same genera have been studied for their anticancer properties, but there are no reports on the Indian species of *Blumea*. Studies have reported that *Blumea balsamifera* showed anticancer activity against human cancer

cell lines (KB, MCF-7, and NCI-H187) [12]. However, the dihydroflavonol extracted from *B. balsamifera* showed abrogation of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) resistance in Adult T-cell leukemia/lymphoma leukemia (ATLL) cell lines [13]. The methanolic extracts of the plant, when tested with hepatocellular carcinoma cell line (McA-RH7777) and Hep-G2, was found that the cell growth was restricted at the G1 phase of the cell cycle in a dose-dependent manner, which affected the DNA replication in Hep-G2 cells and decreased the unprocessed and soluble form of a proliferation-inducing ligand (APRIL) [14]. The effect is mainly caused by the decrease of cyclin-E synthesis and the ratio of hypo-phosphorylated versus hyper-phosphorylated of retinoblastoma (Rb) gene.

Blumea lacera has not yet been studied for its antioxidative properties and anticancer properties. In the current paper, the antioxidative potential and the anticancer capacity of the plant leaves in fresh and dry forms were evaluated.

Materials and methods

Sample collection and processing

The plant material was collected in August from the Ulve area, Maharashtra, India. Botanical authentication was done by an expert in the field. The plants were selected from the area that was away from urbanization to reduce the level of surface pollutants.

The leaves from the plant were first washed thoroughly and air dried at room temperature. The fresh samples were prepared after overnight drying while the dry samples were prepared by drying for 5 days. The drying of leaves was carried out in a clean and well-aerated room. The leaves were crushed in a mortar pestle by using liquid nitrogen to produce a very fine powder.

The initial extraction was done by using 5 g of the powdered leaves mixing with the respective solvents (hexane followed by methanol and ethanol). The extraction was carried out overnight on a shaker at the speed of 150 rpm. After overnight extraction, the extract was filtered through a muslin cloth to remove debris. The filtered extract was concentrated by using BUCHI Rotavapor™ R-100 Rotary Evaporator Systems (Fisher Scientific, Pittsburgh, PA, USA). The final concentration of the extract was made up to 5 g/mL [15]. The methanolic extract obtained from the fresh sample was labelled as BMF (*Blumea* Methonalic extract fresh sample) and the dry sample was labelled as BMD (*Blumea* Methonalic extract dry sample). For the ethanolic extracts, the fresh sample was labelled as BEF (*Blumea* Ethanolic extract fresh sample) and the dry sample was labelled as BED (*Blumea* Ethanolic extract dry sample).

Total phenolic content (TPC) assay

The TPC was assessed using the Folin-Ciocalteu method [15]. The methanolic and ethanolic extracts were prepared at different concentrations (5, 10, 15, 25, 30, and 35 mg/mL). Then, 100 µL of Folin-Ciocalteu reagent and 300 µL of aqueous sodium carbonate were added to 20 µL of each concentration. The samples were kept at room temperature for 30 min and then the absorbance was measured at 650 nm, spectrophotometrically. The standard curve was plotted using gallic acid as the standard. TPC was expressed as mg gallic acid standard equivalent per gram of dry extract. All the experiments were performed in triplicate. Correlation coefficients to estimate the relationship between two variables (between different tests and content of total phenolic and flavonoid compounds) were calculated using Microsoft Excel software.

Total flavonoid content (TFC) assay

The TFC was determined as per previously described method [16, 17]. 0.5 mL of various dilutions of the plant extract was added to 0.5 mL of 2% aluminium chloride. This mixture was incubated for 10 minutes, and the absorbance was measured spectrophotometrically at a wavelength of 368 nm. The amount of flavonoids in the extracts was determined using quercetin as

a standard flavonoid compound. The samples were analyzed in triplicates.

1,1-diphenyl-2-picrylhydrazyl (DPPH) assay

The free radical scavenging activity was analyzed using DPPH assay [15]. 0.3 mM DPPH was prepared in methanol and 2 mL of DPPH was mixed with 200 µL of different concentrations of plant extracts prepared in methanol and ethanol. The reaction mixture was incubated in dark at room temperature for 30 minutes. The absorbance of the mixture was measured at 515 nm. All the determinations were performed in triplicate. The DPPH radical scavenging activity was calculated as follows:

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

Thiobarbituric acid reactive substances (T-BARS) assay

A method was followed to determine the % protection provided by the plant extracts against oxidative stress induced by the ascorbate- Fe^{2+}-system [15]. The formed T-BARS were measured at 532 nm as malondialdehyde (MDA) equivalents. The preparation of standard was done by acid hydrolysis of tetraetoxypropane. 0.5 mL of plant extract (100 mg/mL) was added to 0.5 mL of 2% $AlCl_3$. The mixture was incubated for 10 minutes and record the absorbance at 368 nm.

Ferric-reducing antioxidant power (FRAP) assay

The method described earlier was utilized for determining the ferric reducing potential [18-20]. The FRAP reagent (0.3 mM acetate buffer (pH 3.6), 10 mmol 2,4,6-Tripyridyl-S-triazine (TPTZ) solution (prepared in 40 mM HCl), and 20 mM ferrous chloride in the proportion of 10:1:1 (v/v)) was prepared freshly. 900 µL of FRAP reagent was added to 90 µL of distilled water and 30 µL of the sample. The absorbance was measured at 595 nm after 5 minutes. All the determinations were performed in triplicate.

High performance liquid chromatography (HPLC)

To determine the phenolic compound content in the plant extracts an HPLC analysis was carried out on the Waters HPLC (Model 2487) (Waters Corporation, Milford, MA, USA) by using a reversed phase hypersil C18 column with a cut off at 5 µ particle size. 25% and 75% methanol were used in 1% acetic acid as the mobile phase and the flow rate was maintained at 75 mL/min. Elution was carried out in a gradient fashion starting with the 25% methanol mobile phase and ending with the 75% methanol-based mobile phase using 280 nm wavelength for detection. The retention times of the different phenolic compounds were used for their identification by comparing to the chromatogram of the standard. The standards for the phenolic compounds were obtained from Sigma (Saint Louis, MO, USA). The peak area measurement was used to determine the concentration of each compound.

Cell culture

Cell lines were procured from NCS (Pune, Maharashtra, India). Melanoma cell line (B16F10) was kindly provided by Dr. Gude from The Advanced Centre for Treatment, Research and Education in Cancer (ACTREC) (Navi Mumbai, Maharashtra, India). Cells were routinely passaged in Iscove Modified Dulbecco Media (IMDM) (Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS) and penicillin (100 U/mL) and streptomycin (100 mg/mL) procured from Himedia Labs (Mumbai, India). Cultures were maintained in an incubator at 37°C in 5% CO_2.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

The method developed earlier was used for detecting the cytotoxicity of the extracts against the melanoma cell lines. The cells were plated on 96 well plate at 4×10^4 cells per well [21]. After allowing the cells to adhere to the wells, the plant extracts were added at various dilutions and incubated at 37°C for 24 and 48 hours. After completion of the treatment, the wells were given a wash with PBS and incubated for 4 hours after the addition of MTT, followed by the addition of DMSO. The absorbance was then

recorded at 540 nm using a microplate reader (Biotek ELx 800) (BioTek, Winooski, VT, USA).

Wound-Healing Assay

The melanoma cells were seeded on 35 mm plates at 2×10^4 cells per plate. After complete cell confluency was achieved, the cells were then treated with the plant extracts in the IMDM medium for a duration of 24 and 48 hours. A pipette tip was used to scratch the monolayer cells with an equal amount of pressure, followed by a wash with PBS. After 72 hours incubation, cells were fixed using methanol and then stained with 0.5% crystal violet. Samples were analyzed in triplicates.

Colony formation assay

B16F10 cells were seeded at 500 cells per dish in petri dish of 60 mm diameter and incubated at 37°C for 24 hours. The cells were next subjected to treatment with the different dilutions of plant extracts for a duration of 24 and 48 hours followed by a wash with PBS. The cells are allowed to grow for 72 hours after which the cells were fixed with methanol. The cells were then stained with crystal violet [22]. Samples were analyzed in triplicates.

Results

Total phenol assay (TPC)

Total phenolic content was analyzed using Folin-Ciocalteu reagent [15]. The evaluation of the phenolic content was expressed as microgram gallic acid equivalent per gram of dry extract. Phenols exhibit antioxidant activity and therefore the measure of phenol gives an estimate of the extracts' antioxidant activity. The experimental analysis revealed that the highest total phenolic content was 100.96 GAE mg/mL for BMF and 111.13 GAE mg/mL for BMD. The results demonstrated that the methanolic extracts showed a much higher concentration of phenolic content as compared to the Ethanolic extracts. The lowest concentration of the methanolic extracts also exhibited a much higher amount of gallic acid as compared to the Ethanolic extracts. The highest phenolic content was observed in methanolic dry extract, while the lowest content was observed in the ethanol dry sample (Table 1).

Alcoholic solvents are known to extract phenolics from natural sources where they show a high yield of total extract [23]. The difference in the phenolic content may be due to the different solvent systems used for the extraction process [24]. Methanol was found to be a more suitable solvent for the extraction of phenolic compounds as seen in various studies [25, 26]. The ability of methanol to cause inhibition of polyphenol oxidase that brings about the oxidation of phenolics, and its ready evaporation compared to water was studied earlier [27]. Therefore, water is a suitable choice for the solvent while extracting phenolic compounds.

Total flavonoids assay

The evaluation of the flavonoid content in the sample is carried out in terms of mg quercetin equivalent/g of dry extract. Flavonoids exhibit antioxidant activity and, therefore, the measure of flavonoids gives an estimate of the antioxidant activity of the extract. The highest flavonoid content was found to be in the BMF (222.6 mg quercetin equivalent/g dry extract) and in the BMD extract (291.66 mg quercetin equivalent/g dry extract) (Table 2). The results demonstrated that the methanolic extracts showed a much higher concentration of flavonoid content as compared to the ethanolic extracts. The results for flavonoids are also congruent with the results for total phenolic content and DPPH, however, as stated earlier a direct correlation is not recommended [28]. The earlier results have also claimed that the methanolic extracts showed better extraction of flavanoids as compared to the ethanol solvent system [29].

DPPH assay

The tabulated results indicate the percent inhibition that the sample possesses. Thus, the IC_{50} indicates the 50% activity of the plant extracts i.e., 50% of the total antioxidative potential of the plant. The IC_{50} for methanolic extracts lied between 3-4 mg/mL, while for the

Table 1. TPC assay results showing the phenolic content in terms of mg Gallic acid equivalent/g dry sample (mg GAE/g) of the methanolic and ethanolic extracts. The methanolic samples showed a higher phenol content in both fresh and dry extract as compared to that of ethanolic samples.

Sample	5 mg/mL	10 mg/mL	15 mg/mL	20 mg/mL	25 mg/mL	30 mg/mL	Mean TPC (mg GAE/g)
BMF	34.71	49.29	60.54	78.45	87.21	100.96	68.53
BMD	62.63	78.88	86.38	88.04	104.29	111.13	88.56
BEF	28.46	29.71	35.13	45.13	48.88	53.88	40.20
BED	39.71	39.71	41.79	43.46	43.88	46.38	42.49

Table 2. TFC assay results showing the flavonoid content in the methanolic and ethanolic extracts. The methanolic samples showed a higher phenol content in both fresh and dry extract as compared to that of ethanolic samples.

Sample	5 mg/mL	10 mg/mL	15 mg/mL	20 mg/mL	25 mg/mL	30 mg/mL
BMF	58.88	60.02	85.99	107.04	125.97	222.61
BMD	101.06	149.82	184.40	245.80	277.53	291.66
BEF	45.29	49.77	59.22	66.19	88.38	91.36
BED	59.22	78.33	88.83	107.48	120.42	134.85

Table 3. DPPH assay results showing the free radical scavenging activity for the methanolic and ethanolic extracts. The IC_{50} values for the extracts indicated the superiority of the radical scavenging activity of methanolic extracts over the ethanolic extracts.

	% Inhibition (Scavenging capacity)						
Sample	**5 mg/mL**	**10 mg/ml**	**15 mg/mL**	**20 mg/mL**	**25 mg/mL**	**30 mg/mL**	**IC_{50} (mg/mL)**
BMF	61.12	63.56	64.33	66.18	67.55	63.44	3 - 4
BMD	47.56	48.52	49.26	50.38	51.36	53.69	3 - 4
BEF	47.10	47.92	48.26	48.40	48.50	52.80	25 - 30
BED	49.23	49.83	49.90	51.30	54.49	49.79	15 - 20

ethanolic extracts the IC_{50} from 25-30 mg/mL for fresh sample and 15-20 mg/mL for dry ethanolic extract (Table 3). Thus, the radical scavenging capacity for methanolic extracts is much higher in comparison to the ethanolic extracts. These results can be correlated with the results for total reducing capacity. However, this theory was not supported by some studies which state that it is not necessary to correlate antioxidant activity only with the high amounts of phenols [28, 30-33]. The methanolic system of solvent seems to have a better activity as compared to ethanol, as the methanolic extracts show better activity. However, this cannot be considered as conclusive as a single system may be erroneous and other tests are recommended [34].

T-BAR assay

The table 4 represents the inhibitory effect of the plant extract in different solvents on lipid peroxidation in rat liver mitochondria. The BMF extract has the highest percent protection of 60.04% followed by the BMD extract having percent protection of 51.25% (Table 4). The increase in antioxidant activity increases percent protection against lipid peroxidation.

Table 4. T-BARS assay results for the methanolic and ethanolic extracts. A higher % protection of the cells was observed for the cells treated with methanolic dry leaf extract as compared to that of the other plant extracts.

Plant Extract	% Protection
BMF	60.04
BMD	51.25
BEF	59.79
BED	55.54

Table 5. FRAP assay results indicated the higher reducing ability of the methanolic extract as compared to that of ethanolic extract as indicated by the higher absorbance values.

Sample	5 mg/mL	10 mg/mL	15 mg/mL	20 mg/mL	25 mg/mL	30 mg/mL
BMF	0.002±0.0010	0.003±0.008	0.003±0.010	0.010±0.014	0.010±0.010	0.010±0.017
BMD	0.003±0.0020	0.004±0.014	0.004±0.011	0.004±0.006	0.005±0.038	0.006±0.013
BEF	0.002±0.0010	0.002±0.000	0.002±0.007	0.003±0.019	0.003±0.013	0.003±0.002
BED	0.002±0.0015	0.002±0.003	0.002±0.017	0.003±0.004	0.003±0.023	0.003±0.032

Table 6. HPLC analysis to determine the polyphenol content in leaf extract. Methanolic extracts showed a higher polyphenol content.

Sample	Vanillin (µg/5g of sample)	Tannic acid (µg/5g of sample)	Catechol (µg/5g of sample)	Caffeic acid (µg/5g of sample)
BMF	-	10.04	58.73	-
BMD	-	10.67	86.65	22.23
BEF	1.07	-	-	-
BED	-	-	-	-

FRAP assay

FRAP measures the ferric reducing ability of the antioxidant molecule at a low pH. The change in absorbance is a direct correlation to the sample reducing capacity [35]. The methanolic extracts showed a higher reducing ability as compared to that of ethanolic extract. The BMF extract showed the highest activity with an absorbance of 0.007, and the BMD showed the absorbance of 0.0062 at 30 mg/mL (Table 5). Previous studies also claimed that the methanolic extracts were more efficient in the reducing ability for plant extracts [36].

HPLC

The methanolic extracts showed the presence of more polyphenolic standards as compared to that of ethanolic extracts. The ethanolic fresh extracts showed the presence of only vanillin while the dry ethanolic extract did not show the presence of any of the tested standards. The methanolic extracts showed the presence of Tannic acid and Catechol. The dry methanolic extract exhibited a higher amount of polyphenols as compared to that of fresh extract (BMF: Tannic acid 10.04 µg/5g of sample and Catechol 58.73 µg/5g of sample; BMD: Tannic acid 10.67 µg/5g of sample, Catechol 86.65 µg/5g of sample, and Caffeic acid 22.23 µg/5g of sample) (Table 6).

MTT assay

The cytotoxicity profile was assessed for all the extracts and the profile was found to be similar for both methanolic and ethanolic solvent systems on the melanoma cell lines. Therefore, a comparison was made for only the methanolic extracts at 24 and 48 hours.

The IC_{50} of the methanolic extracts in both fresh and dry forms was found to be at 1,500 µg/mL in the 24 hours study; while in the 48 hours study the IC_{50} was 1,000 µg/mL. The ethanolic extracts showed an IC_{50} of 1,300 µg/mL for 24 hours study, while the 48 hours study found IC_{50} to be around 700 µg/mL for the fresh extracts and 1,000 µg/mL for the dry extracts (Figure 1).

Colony formation assay

The anti-proliferative effect of the extract was further assessed by performing a colony formation assay. The results were found to be seen in Table 7. These results depicted the anti-proliferative effect of the extracts over the melanoma cell line. The dry extract showed a higher efficiency than that of fresh extract as is demonstrated in the 24 hours study (Dry extract at 100 µg/mL showed 47.82% inhibition, while the fresh extract showed 36.95% inhibition at 100 µg/mL). The fresh extract showed a possible

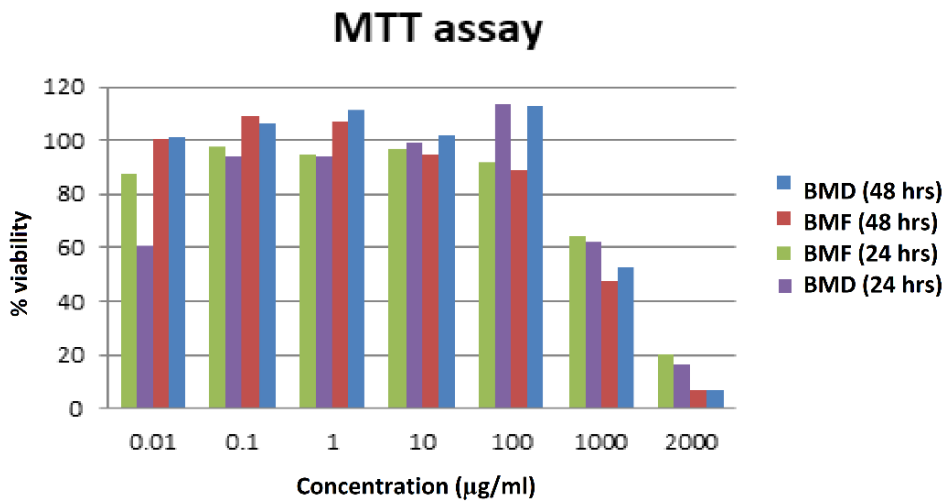

Figure 1. The cytotoxicity of the plant extracts against B16F10 cell lines using MTT assay.

Table 7. Colony formation assay results. The table depicts the capacity of the plant extracts to inhibit the colony forming capacity of the B16F10 cell. The analysis was carried out for methanolic samples only. The % inhibition was found to be higher for the dry leaf extracts.

	% Inhibition					
	BMF			BMD		
	1 µg/mL	10 µg/mL	100 µg/mL	1 µg/mL	10 µg/mL	100 µg/mL
24 hrs	8.69±8.84	34.78±3.68	36.95±4.79	28.26±2.08	47.82±1.25	47.82±4.27
48 hrs	21.87±4.92	9.37±5.71	9.37±2.38	9.37±4.04	9.37±5.44	28.12±2.36

Table 8. Wound scratch assay results showed a better effect when the treatment was given for 48 hr.

	% Inhibition					
	BMF			BMD		
	1 µg/mL	10 µg/mL	100 µg/mL	1 µg/mL	10 µg/mL	100 µg/mL
24 hrs	54.41±5.88	64.06±5.18	100.00±0.00	61.01±5.71	68.8±3.60	75.37±2.95
48 hrs	46.05±5.51	56.18±4.51	69.21±2.80	47.04±0.00	63.2±2.64	72.16±2.80

biphasic effect in a time-dependent manner, as the anti-proliferative activity seemed to be reversed at higher doses. Further analysis is required to determine the biphasic effect of the extracts.

Wound scratch assay

The anti-migratory effect of the plant extracts against the melanoma cell line was assessed using the wound scratch assay and the superiority of dry extract in preventing migration of the cancerous cell lines was observed through the data in the Table 8. The cells exposed to the plant extract for 24 hrs showed a better activity than that of the 48 hrs study. Dry extract at 100 µg/mL showed 75% inhibition, while fresh extract showed a 100% inhibition at 100 µg/mL in the 24 hrs treatment.

Discussion

Melanoma is a form of skin cancer that has been on a steady increase for many years [37]. Many of the synthetic drugs used for the treatment pose the problem of damaging side effects and

hence it becomes necessary to explore alternative strategies. Another cause of concern is the resistance against the existing drugs in the market [38]. Phytochemicals have been investigated for their anticancer potential by targeting various pathways in the cancerous cells and the results have shown great potential [39]. The advantages of plant-based anticancer therapeutics become evident while considering the possibility of having these therapeutics as a part of the human diet and, therefore, the chance of acceptability in the population increases [40]. The current study demonstrates the use of *Blumea lacera* as an antioxidant and anticancer agent by using the extract produced from the plant leaves using methanol and ethanol as a solvent. *Blumea lacera* is an herb with many applications in traditional Indian medicine and, therefore, the antioxidant and anticancer activity of the alcoholic extracts of the leaves was investigated [2, 3]. The methanolic extract of the leaves (fresh and dry extracts) showed an effective antioxidant activity as compared to that of ethanolic extract. Also, the methanolic extract showed a better anticancer activity than that of ethanolic extracts. The fresh and dry forms of the extracts also showed a significant difference in their activities. The study thus demonstrates the higher activity of the dry methanolic extracts of the leaves and warrants a further study on the extracts from different plant parts. The phytochemical analysis revealed that the methanolic fraction had a higher composition of polyphenols and this is also reflected in the antioxidant capacity of the methanolic extracts. The polyphenols are known for their antioxidant and anticancer ability and the special interest in assessing the polyphenol-based activity stems from their abundance in the food sources [41]. This strategy has huge promise in the field of therapeutics and drug development as the phytochemicals obtained from the methanolic fraction can be further studied to develop an efficient therapeutic.

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