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Research Article

VALORIZATION OF ANTIOXIDANT, ANTICANCER ACTIVITIES, AND PHYTOCHEMICAL ANALYSIS OF ETHANOLIC EXTRACT OF *EUPHORBIA CUNEATA* VAHL., AND ITS EFFECT ON THE EXPRESSION OF P53, RAF-1AND CASP3 GENES

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ABSTRACT

Objective: *Euphorbia cuneata* Vahl. (*E. cuneata*) is used in traditional medication to relieve inflammation and pain, while the biological foundation of these actions has not been fully explored.

The purpose of this work was to explore the pharmacological characteristics and classification of flavonoid and phenolic constituents found in aerial parts of *E. cuneata*.

Methods: *E. cuneata* was examined for cytotoxic effects, antioxidant activity, and cell viability. The flavonoid and phenolic contents, and fugacious constituents were also characterized; furthermore, the expression levels of Raf-1, P53, and Casp3 genes using qRT-PCR method were investigated.

Results: Ethanolic extract (EE) of *E. cuneata* showed the highest antioxidant activity, the antioxidant activity increased from (25.12) at 10 µg/mL to (97.90) at 1280 µg/mL. The EE has an IC_{50} of 28.52 µg/mL. *E. cuneata* EE extract had strong cytotoxic effects on human cell lines of lung cancer (H1299), breast cancer (MCF7), and colon cancer (HCT-116), respectively according to the MTT test; with lower IC_{50} (125.01, 149.56 and 148.56) µg/mL, respectively. The most common phenolic acid identified in the EE extract of *E. cuneata* was pyrogallol, in addition, the most abundant flavonoid was found to be 7-hydroxyflavone; on the other hand, GC mass analysis showed that the EE extract was rich in methyl 12-hydroxy-9-octadecenoate. Treatment of H1299 with the IC_{50} of EE resulted in a considerable downregulation of Raf-1 gene and upregulation of P53 and Casp3 genes.

Conclusion: We may infer that the EE extract of *E. cuneata* includes natural bioactive components, moreover antioxidant and anticancer characteristics, which may have therapeutic potential.

Keywords: *Euphorbia cuneata*, Antioxidant, Cytotoxicity, Gene expression.

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INTRODUCTION

Plants are highly rich sources of bioactive molecules useful for a multitude of applications in the fields of biomedicine and food-related purposes. Infections caused by microorganisms and the growth of drug-resistant microbes have become one of the world's most grave problems. Due to the imperfect number of effective medications and the negative effects of synthetic antibiotics, microbial infections kill millions of people each year [1, 2]. Bioactive substances found in medicinal plants consist of a wide range of phytochemicals with different action mechanisms. Several phytochemicals have been found to have significant antimicrobial properties [3]. On the other side, cancer is a very important health problem and a major cause of death on a large scale. Cancer is a multistage disease that begins with genetic changes and progresses through the growth of aberrant cells [4]. Globally, lung cancer accounts for 11.6% of all cancer cases, followed by breast cancer coming at 11.6%, prostate cancer at 7.1%, and colorectal cancer at 6.1% [5]. Although a lot of work has gone into creating effective chemotherapy drugs, problems with toxicity and selectivity still persist. Researchers are searching for alternative therapies and prevention measures due to the toxicity of existing chemotherapy and the resilience of cancer cells to anticancer medications. Natural plant products might be able to help. One of the largest flowering plants families, the Euphorbiaceae family has over three hundred genera and eighty thousand species, and they may be found in a wide range of habitats [6].

According to Mwine and Van Damme [7], several species of Euphorbiaceae have a tendency to produce a broad variety of secondary metabolites to help them respond to a variety of stimuli in their specific environment.

E. cuneata, also known as *Euphorbia cuneata* Vahl., is indigenous to the Arabian Peninsula and extends southward through Sudan, Ethiopia, and the coastal areas of Somalia, Kenya, and Tanzania. It is also grown and occasionally escaped in Mozambique and is utilized in a variety of traditional civilizations [8, 9].

In South America, Asia, and Africa, plants belonging to the genus *Euphorbia* are found in moderate, tropical, and subtropical climates. *E. cuneata* has been shown to contain a variety of active ingredients, including: flavonoids (cuneatannin) and triterpenes (cyclocuneatol) [10], but lacks the harmful diterpenes found in other *Euphorbiaceae* species [11].

A current approach is to use new technology to identify, separate, and apply biologically active compounds to specific illnesses, therefore leveraging traditional medicine's knowledge base. Pathologies that cause pain and inflammation have recently sparked a lot of interest in potential therapeutics based on physiologically active compounds extracted from medicinal plants [12].

This study's objectives were to assess the antioxidant and anticancer properties of *E. cuneata* aerial component extracts and to use GC-mass and HPLC to analyze phenolic acids and volatile compounds. Hence, the goal of this study was to decide the antioxidant and anticancer activities of different *E. cuneata* leaf extracts against three cell lines: H1299, HCT-116, and MCF7 cell lines, as well as to determine the chemical composition of ethanolic extract (EE) of *E. cuneata* using HPLC and GC-mass to analysis phenolic acids and volatile compounds; furthermore, to study the expression levels of Raf-1, P53, and Casp3 genes using qRT-PCR method.

METHODS

Chemicals

All high-quality analytical grade chemicals and HPLC standards utilized were acquired from Sigma Chemical Co.

Preparation of sample

Libya's Al-Jabal Al-Akhadar regions provided the *E. cuneata* plants used in this study. After being cleaned, the plant's aerial portions were left to dry in the shade. Before being employed in further research, the leaf was finely milled to a powder and kept in screw-capped vials.

Extraction procedure

Separately, 10 g of the powdered material were shaken at 200 rpm for 24 h at RT in 100 mL of solvent (water, ethanol, toluene, and *n*-hexane) according to the polarity index. Using Whatman filter paper, the mixture was filtered. After re-extraction with 50 mL of solvent, the residue was filtered, and the all fractions were collected and concentrated at low pressure using a rotary evaporator. To reach a standard concentration of 100 mg/mL, the resultant residue was dissolved in the least amount of solvent feasible. Before being used, extracts were kept at −4°C.

Antioxidant activity assay

The DPPH test [13] was used to evaluate the free radical's capacity for scavenging. In this test, the violet color of DPPH becomes pale-yellow when hydrogen atoms are removed from the antioxidant molecule. The reaction combination contained 40 µL of extract at different strengths made by diluting with extraction solvent, as well as 3 mL of a methanol solution of 0.1 mM DPPH solution. After giving the mixture a good shake, it was incubated at 37°C for 30 min. A spectrophotometer was used to detect the absorbance at 515 nm. Ascorbic acid was used as a positive control. The link between stronger free radical scavenging activity and decreased absorbance of the reaction mixture was computed using the following equation:

the percent of DPPH scavenging activity = $100 \times (A_0 - A_1)/(A_0)$.

A_0 and A_1 are the absorbances of the control and sample, respectively. The key values and the standard deviation (SD) are displayed with the average of three duplicate studies. GraphPad Prism software was used to estimate the 50% inhibitory concentration (IC_{50}), the concentration essential for 50% DPPH radical scavenging activity.

Cytotoxic effects

Cell lines

Lung cancer (H1299), colon cancer (HCT-116), and breast cancer (MCF7) human cell lines from the American Type Culture Collection (ATCC), USA, were utilized.

Cell culture

The cells were cultured in Dulbecco's modified Eagle's medium, which was enhanced with 50 µg/mL gentamycin, 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and HEPES buffer. Every cell line was cultivated 2 times a week at 37°C in a humidified atmosphere with 5% CO_2.

Cytotoxicity assay

Mosmann [14] was used to estimate the cytotoxicity test. 100 µL of growth media was used to seed cells in a 96-well plate at a concentration of 1×10^4 cells per well. After 24 h of seeding, fresh medium with varying EE concentrations was applied. Confluent cell monolayers dispersed in a 96-well microtiter plate were treated with serial twofold dilutions of EEs. In a humidified incubator with 5% CO_2, the microtiter plate was incubated for 24 h at 37°C.

For every concentration of the test sample, three wells were employed. In addition to extract, control cells were treated with or without DMSO (dimethyl sulfoxide). The experiment was shown to be unaffected by the little amount of DMSO (0.1%) in the wells.

The colorimetric method was used to measure the number of live cells after the cells were kept for 24 h at 37°C. After the incubation time ended, the medium was aspirated, and each well received 1% crystal violet solution for at least half an hour.

After the stain was eliminated, water was used to rinse the plates. Following a thorough mixing and addition of 30% glacial acetic acid to each well, the absorbance of the plates was calculated at 490 nm using a microplate reader. The number of viable cells was ascertained by measuring absorbance, and the relative viability ratio was computed as $(OD_T/OD_C) \times 100$; where OD_T represents the treated cells' absorbance and OD_C represents the control cells' absorbance. Using the dose-response curve equation, the half maximum inhibitory concentration (IC_{50}) was determined.

Identification of phenolic and flavonoid compounds

HPLC (Agilent 1100) was utilized to recognize the flavonoids and phenolic acids in the EE of *E. cuneata*; prerequisites comprised an Agilent ChemStation, LC pumps, a ultraviolet/visible detector, and a C_{18} column (160 × 4.30 mm, 5 µm particle dimension).

The phenolic acids were separated using a grade mobile phase that included two solvents: Solvent A (methanol), and solvent B (acetic acid in water, 1:25). The column was eluted using the gradients: 0–3 min of solvent B, 5 min of 50% solvent A, 2 min of 80% solvent A, and 5 min of 50% solvent A. Flavonoids were separated using a gradient mobile phase with an isocratic elution technique (70:30) and two solvents, solvent A (acetonitrile) and solvent B (0.2%, v/v formic acid) [15,16].

The extract was diluted with methanol before to injection. The flow rate was set at mL/min. To identify the substances, retention durations and absorption spectra were compared to standards analyzed at 280 and 320 nm for flavonoids and phenolic compounds, respectively.

Volatile components determination

The NIST MASS SPECTRAL database and WILEY were used to identify the compounds. *E. cuneata*'s EE was analyzed by GC-MS using a Thermo Scientific TRACE 1310 gas chromatograph and a single quadrupole mass spectrometer (ISQ LT). J and W Scientific utilized a DB5-MS column that was 30 m long and had an interior diameter of 0.25 mm. Helium served as the carrier gas, flowing at a rate of 1.0 mL/min.

Maintain at 40°C for three min. After that, raise the temperature to 280°C at a pace of 5.0°C/min and hold it there for 5 min. After that, raise the temperature to 290°C at a pace of 7.5°C/min and hold it there for one min. The injection and detection temperatures were 200°C and 300°C, respectively. Using a spectral range of 40–450 m/z, mass spectra were obtained at 70 eV using electron ionization. Using the WILEY and NIST mass spectrometry database, compounds were found.

The concentration of the compounds was determined using the typical calibration curve, and the identities of components were verified by mass spectrometry and retention of the genuine compounds obtained under the same GC-MS circumstances.

Detection of expression levels of Raf-1, P53, and Casp3 genes

qPCR was utilized to examine variations in the mRNA levels of apoptotic-related genes Raf-1, P53, and Casp3 in IC_{50}-treated and untreated H1299 cells, as was previously documented [17]. H1299 cells were exposed to IC_{50} concentrations for 48 h. RNA extraction was performed according to the manufacturer's instructions for TRIzol reagent [18].

Gene quantification by qRT-PCR

A µg of isolated RNA underwent reverse transcription. The prior procedure [19] was followed in the preparation of a real-time PCR master mix for genes linked to apoptosis. The findings from the target mRNA level were extrapolated to the *b*-actin mRNA level. The findings were displayed as a fold change compared to the negative control.

Statistical analysis

Analysis of variance was performed on all of the data. The SD and major values were calculated for each item using three samples. Duncan's multiple range tests were used to determine if the signify differences variables were significant ($p \leq 0.05$). SPSS 16 was used for all analyses [20].

RESULTS AND DISCUSSION

Antioxidant activity of extracts

Table 1 shows that all *E. cuneata* extracts were scavenged the DPPH radical. We found that EE had the maximum scavenging power (97.90%), followed by WE (55.10%), *n*-HE (43.21%), and TE (22.23%). The scavenging efficacy was shown to rise as the extract concentration rose. The IC_{50} values for WE, EE, TE, and *n*-HE were, respectively, 51.61, 28.52, 30.57, and 128.15 µg/mL, as revealed in Figs. 1 and 2.

Significant alterations in extracts were found using statistical analysis. Ascorbic acid was used as a positive control in the DPPH technique to assess antioxidant activity. The amounts of ascorbic acid varied between 1280 and 2.5 µg/mL. Ascorbic acid exhibited an inhibitory rate of 34.57% at 2.5 µg/mL and 99.23% at 1280 µg/mL.

Munro *et al*. [21] found that at all concentrations examined using the DPPH assay, the methanolic extract of *Euphorbia tirucalli* exhibited superior free radical scavenging efficacy compared to the aqueous extract. The polar extract of *E. terracina* L. showed a variety of active secondary metabolites, with saponins and phenolics being the most prevalent, according to El-Amier *et al*. [22].

Anti-proliferation effects on H1299, HCT-116, and MCF7 cell lines

The cytotoxicity of *E. cuneata*'s EE was tested on distinct cancer cell lines. The cytotoxicity of the extract to cell lines was assessed using the micro-culture tetrazolium test. Cytotoxic activity was represented as a percent of cell viability in comparison to the control group. Effective dosages were determined using the dose-response curve and a variety of extract concentrations. Three cancer cell lines were used to estimate the effect of the EE to assess the anticancer activity of *E. cuneata*: MCF7, which represents breast cancer, HCT-116, which represents colon cancer, and H1299, which represents lung cancer.

DMSO, which served as the experiment's negative control, had no discernible impact on the cancer cells. This shows that the *E. cuneata* extract, not the DMSO control, is responsible for the effects shown on cancer cell lines.

E. cuneata extract's cytotoxicity test results against a number of cell lines are displayed in Table 2 and Fig. 3. However, the doxorubicin (reference standard) had an IC_{50} of 0.95, 1.93, and 0.64 µg/mL against H1299, HCT-116, and MCF7 cell lines, respectively, whereas the IC_{50} of EE of *E. cuneata* against these cell lines were 125.01, 149.56, and 148.56 µg/mL.

The study's findings demonstrated that *E. cuneata* extract has strong anticancer properties, particularly against the H1299 lung cancer cell line. Cell viability significantly decreased when EE concentrations were compared to the negative control group. At greater doses (1000 µg/mL), the anticancer action was more noticeable than at lower values.

In contrast to MCF7 and HCT-116 cell lines, H1299 cell line may be more susceptible to the extract's effects, indicating that the anticancer

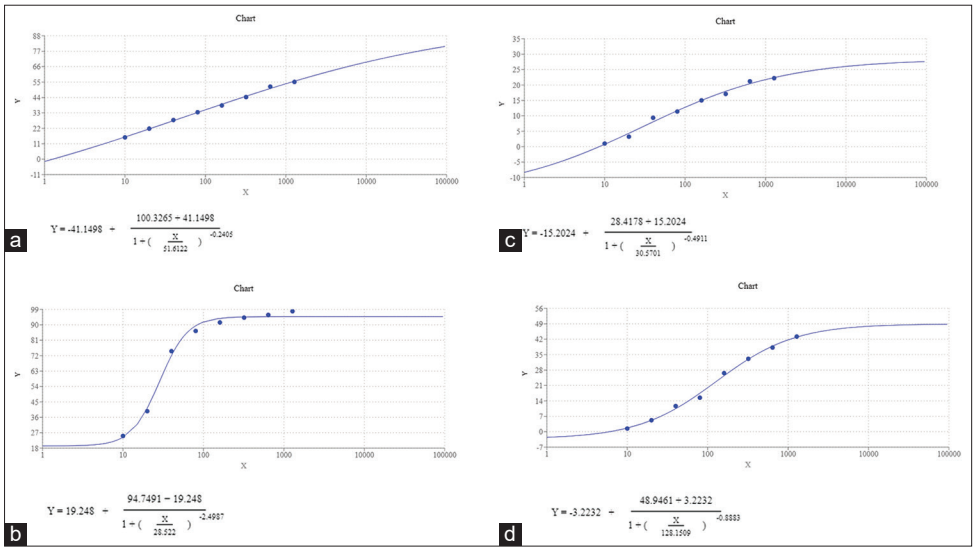

Fig. 1: The IC_{50} value for extracts was determined with a non-linear model. (a) The IC_{50} value for WE; (b) the IC_{50} value for EE; (c) the IC_{50} value for TE; and (d) the IC_{50} value for n-HE. EE: Ethanolic extract

activity of *E. cuneata* may differ based on the particular cancer cell line. Previous research has discovered indications that the active components in *Euphorbia* species may have anticancer properties [23], as well as a relationship between antioxidant activity and the anticancer properties of plant extracts [24].

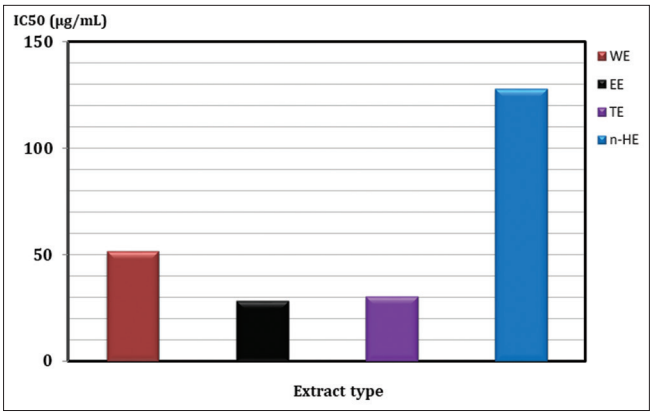

Fig. 2: The IC_{50} values for *Euphorbia cuneata* Vahl. extracts

Table 1: Radical scavenging activity of *Euphorbia cuneata* Vahl. extracts at different concentrations toward DPPH

Radical scavenging activities of extracts*				
Conc. (µg/mL)	**WE@**	**EE**	**TE**	**n-HE**
1280	55.10	97.90	22.23	43.21
640	51.76	95.76	21.21	38.23
320	44.28	94.18	17.10	33.11
160	38.30	91.38	15.03	26.63
80	33.49	86.39	11.40	15.45
40	27.90	74.62	9.37	11.67
20	21.76	39.56	3.25	5.22
10	15.52	25.12	1.02	1.45
IC_{50} value#	51.61	28.52	30.57	128.15

*Radical scavenging activity given as percentage inhibition
@water extract (WE), ethanolic extract (EE), toluene extract (TE), n-hexane extract (n-HE)
#The IC_{50} values for extracts were calculated using GraphPad Prism software (San Diego, CA. USA)
The percentage inhibition value of the standard compound ascorbic acid was 99.23% at a concentration of 1280 µg/mL

Identification of phenolic acids and flavonoids

Different phenolic compounds were identified in the EE of *E. cuneata*, including flavonoids (luteolin, kaempferol, quercetin, rutin, and 7-hydroxyflavone) and phenolic acids (gallic acid, pyrogallol acid, caffeic acid, *p*-coumaric, syringic acid, and ferulic acid). The two most common phenolic acids were caffeic acid and pyrogallol. Next in line were gallic acid, ferulic acid, syringic acid, and *P*-coumaric acid (Fig. 4). According to Fig. 5, the most common flavonoids were rutin (7.62%) and 7-hydroxyflavone (12.45%), which was followed by quercetin, luteolin, and kaempferol.

According to Awaad *et al*. [25], four flavonoids with anti-ulcer potential have been discovered and extracted from alcohol extracts of *E. cuneata* Vahl. These flavonoids include 4'-O-methoxy-luteolin-7-O-rhamnoglucoside, aromadendrin, apigenin, and naringenin. Kebbab-Massime *et al*. [26], Bahar *et al*. [27], and Islam *et al*. [28] have also reported the isolation of naringenin, isoaromadendrin, dihydroquercetin, and isosinensin from *E. cuneata*.

GC-MS analysis of volatile components

GC-MS was used to identify the volatile compositions of the EE of *E. cuneata* leaves based on their mass spectrum, peak area, and retention duration (Figs. 6 and 7). Using GC-MS analysis, five main bioactive compounds in *E. cuneata* were discovered and categorized according to their chemical structures.

Methyl-12-hydroxy-9-octadecenoate (44.39%), hexadecanoic acid, methyl ester (21.34%), hexanal dimethyl acetal (6.59%), methyl octadeca-9,12-dienoate (13.47%), and (9E,12E)-octadeca-9,12-dienoyl chloride (14.21%).

The findings aligned with those of Yener *et al*. [29], who discovered that the main fatty acids extracted from *Euphorbia* species were linoleic acid, palmitic acid, 17-tetratriacontane, and hexatriacontane.

The Euphorbiaceae family has a wide variety of triterpenoids. Numerous skeleton kinds, including euphane, tirucallane, cycloartanes, lanostane, oleanane, lupine, taraxerone, friedelane, friedoursane, and ursane, have been described in the genus *Euphorbia* [30]. The aerial portions of

Table 2: Toxicity effects of EE of *E. cuneata* against different cancer cell lines

Samples Conc. (µg/mL)	EE of *E. cuneata*								
	Cancer cell lines								
	H1299#			HCT-116 and			MCF7@		
	Viability (%)	Inhibitory (%)	S.D. (±)	Viability (%)	Inhibitory (%)	S.D. (±)	Viability (%)	Inhibitory (%)	S.D. (±)
1000	4.77	95.23	0.75	9.66	90.34	1.32	17.14	82.86	0.62
500	15.33	84.67	0.39	20.55	79.45	1.94	23.87	76.13	0.95
250	30.18	69.82	0.87	39.35	60.55	3.71	43.39	56.61	1.83
125	45.65	54.35	1.39	55.18	44.82	2.89	59.55	40.45	2.72
62.5	69.96	30.04	2.42	73.35	26.65	1.73	77.67	22.33	1.98
31.25	84.41	15.59	1.73	92.44	7.56	1.45	92.12	7.88	0.92
15.6	91.52	8.48	0.61	97.02	2.98	0.82	98.48	1.52	0.64
7.8	94.31	5.69	0.54	100	0	0	100	0	0
0	100	0	0	100	0	0	100	0	0

#H1299, human lung cancer cell line;
&HCT-116, human colon cancer cell line;
@MCF7, human breast cancer cell line
E. cuneata: Euphorbia cuneata Vahl., SD: Standard deviation, EE: Ethanolic extract

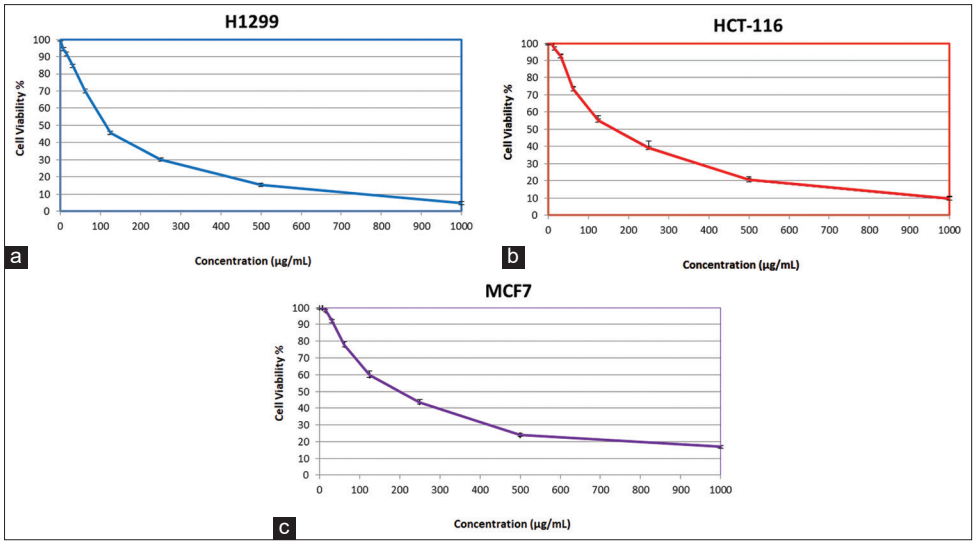

Fig. 3: Toxicity effects of ethanolic extract of *Euphorbia cuneata* Vahl. against different cancer cell lines: (a) H1299, human lung cancer cell line; (b) HCT-116, human colon cancer cell line; (c) MCF7, human breast cancer cell line

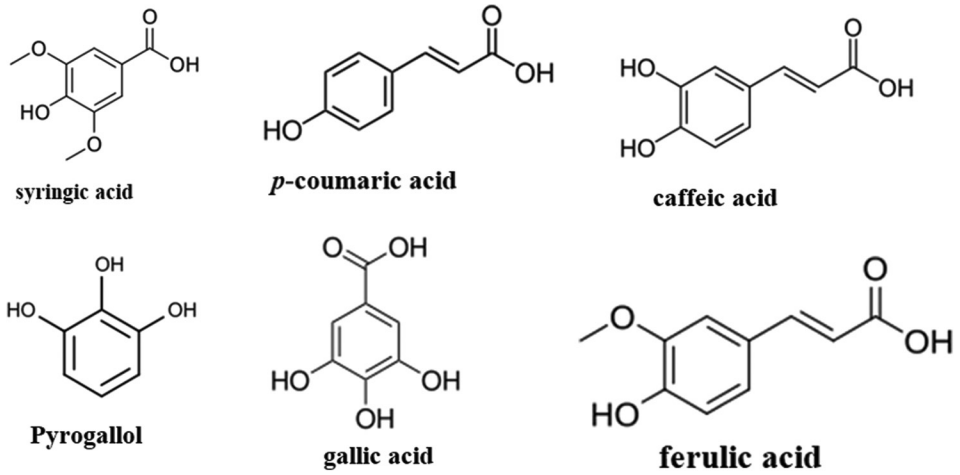

Fig. 4: Chemical structure of the phenolics commonly identified in ethanolic extract of *Euphorbia cuneata* Vahl

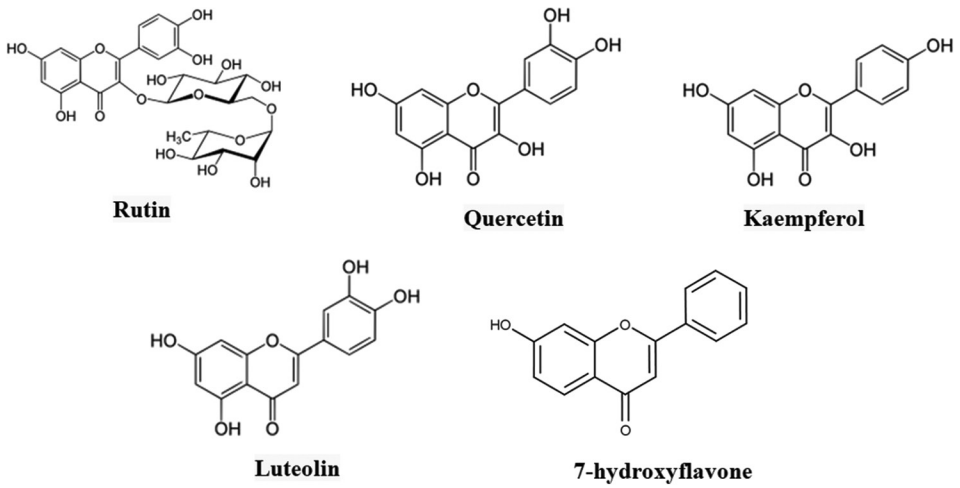

Fig. 5: Chemical structure of the flavonoids commonly identified in ethanolic extract of *Euphorbia cuneata* Vahl

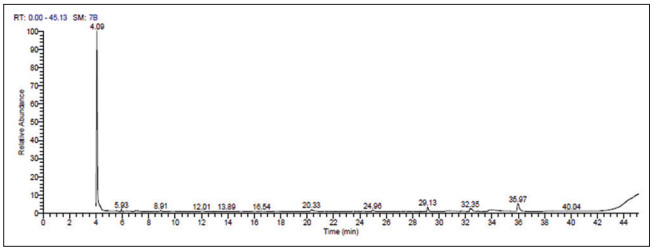

Fig. 6: GC chromatogram of the composition of volatile substances in the ethanolic extract of *Euphorbia cuneata* Vahl. leaves

Euphorbia dendroides L. were used to extract a novel triterpene of the cycloartane type [31].

Euphorbia has been discovered to contain a few sesquiterpenoids [32]. Azizi *et al*. discovered aryanin, a novel sesquiterpene lactone, from the aerial portions of *Euphorbia microsphaera* Boiss [34].

Gene expression analysis

The changes in gene expression level of Raf-1, P53, and Casp3 genes in response to IC_{50} treatment of *E. cuneata* ethanolic leaf extract were evaluated using the qPCR method. qPCR results indicated that treatment

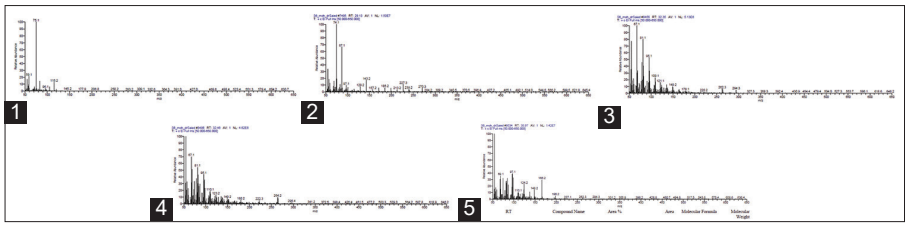

Fig. 7: Mass spectrum of the composition of volatile substances in the ethanolic extract of *Euphorbia cuneata* Vahl. Leaves. (1) hexanal dimethyl acetal; (2) hexadecanoic acid, methyl ester (palmitic acid methyl ester); (3) 9,12-octadecadienoic acid, methyl ester; (4) 9-octadecenoic acid, methyl Ester; and (5) methyl 12-hydroxy-9-octadecenoate

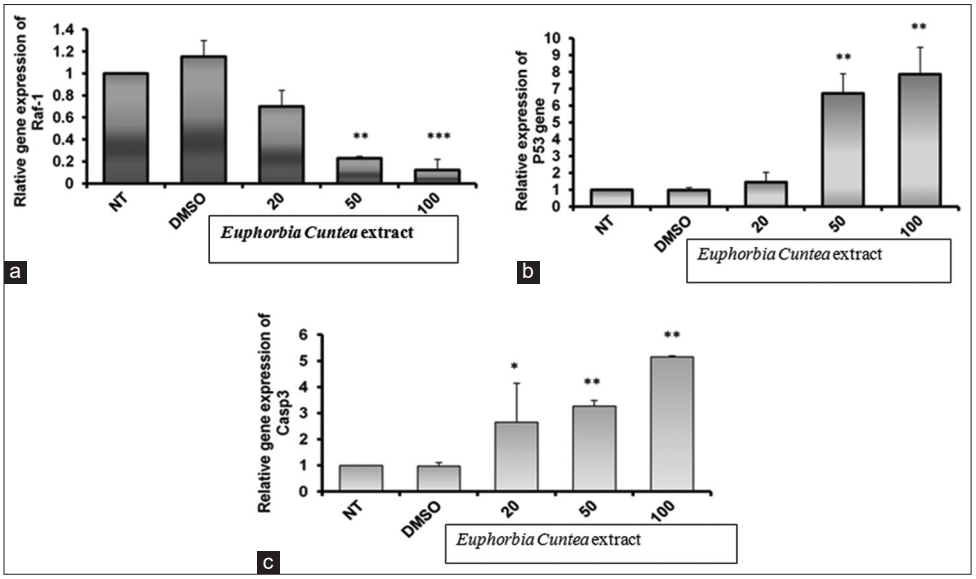

Fig. 8: Changes in mRNA levels of Raf-1 (a), P53 (b), and Casp3 (c) genes among H1299 cell line in response to treatment with IC_{50} of Ethanolic extract of *Euphorbia cuneata* Vahl. in comparison with untreated cell (control) after 24 h. Error bars indicate the standard deviation of three independent experiments

Table 3: Quantification analysis of Raf-1, P53, and Casp3 in SEM-treated cells

Genes	Condition	Expression fold changes	Standard deviation	Student two-tails t-test	p-values
Raf-1	NT	1.00	0.00		
	DMSO	1.15	0.14	0.26	>0.05
	20 µg/mL	0.70	0.15	0.089	>0.05
	50 µg/mL	0.22	0.02	0.046	<0.05*
	100 µg/mL	0.12	0.09	0.006	<0.01**
P53	NT	1.00	0.00		
	DMSO	0.98	0.15	0.86	>0.05
	20 µg/mL	1.43	0.58	0.40	>0.05
	50 µg/mL	6.72	1.17	0.02	<0.05*
	100 µg/mL	7.84	1.60	0.02	<0.05*
Casp-3	NT	1.00	0.00		
	DMSO	0.97	0.14	0.83	> 0.05
	20 µg/mL	2.66	1.48	0.25	>0.05
	50 µg/mL	3.27	0.21	0.004	<0.01**
	100 µg/mL	5.1	0.043	0.001	<0.01**

*Indicates p-values ≤0.05 and **indicates the p ≤0.01.

of H1299 with IC_{50} of EE of *E. cuneata* leads to downregulation of Raf-1 gene. The expression level of Raf-1 gene was significantly downregulated by treatment of H1299 with *E. cuneata*. qPCR results also indicated that treatment of H1299 with IC_{50} of EE of *E. cuneata* leads to upregulation of P53, and Casp3 genes (Table 3 and Fig. 8).

Azizi *et al*. [34] demonstrated that an extract from *E. lathyris* seeds, which are high in polyphenols such esculetin, gaultherin, euphorbetin, and kaempferol-3-rutinoside, had an antiproliferative impact on glioblastoma multiforme and colon cell lines (T84 and HCT15). The scientists showed that the overexpression of caspase-9, 3, and 8 (casp-9, 3, and 8), caspase-3, and autophagy activation are linked to the induction of apoptosis.

CONCLUSION

The present study comes to the conclusion that *E. cuneata* EE is a highly potential source of new nontoxic and antioxidant constituents, and that it contains notable levels of flavonoids, phenolic, and volatile compounds. These findings also give its application in the treatment of cancer a solid foundation. To identify the precise active ingredient in *E. cuneata*'s EE and validate its mode of action, more research is needed.

CONFLICTS OF INTEREST

The authors have no conflicts of interest.

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