ORIGINAL ARTICLE

Gc–ms Analysis and Cytotoxic Evaluation of Cosmos Caudatus Extracts in Oral Squamous Cell Carcinoma (OSCC) Cells

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ABSTRACT

Background: Oral squamous cell carcinoma (OSCC) is the most prevalent and aggressive form of oral malignancy. Despite advancements in treatment modalities, OSCC continues to exhibit high morbidity and mortality rates, highlighting the urgent need for novel therapeutic strategies. Cosmos caudatus (CC), an endemic to Mesoamerica, is reported to retain anticancer potency against various cancer types like cervical, breast, and leukemia. These beneficial properties are linked to its rich phytochemical content. **Methods:** GC-MS analysis was performed on aqueous and methanolic leaf extracts of CC. MTT assay was used to determine the cytotoxic effect of both extracts on HSC-2 cells. **Results:** The GC-MS analysis of crude extracts from both solvents revealed 13 bioactive compounds. MTT assay revealed that both extracts had cytotoxic effects on HSC-2 cells. CCME showed a stronger inhibitory effect on HSC-2 cell line at different time points, 24, 48, and 72 hours with IC50 of 0.26, 0.24, and 0.31 mg/ml, respectively. The CCAE inhibition effect was detected at 48 and 72 hours with IC50 of 2.1 and 0.93 mg/ml, respectively. **Conclusion:** Major compounds retaining bioactive properties were identified from CCAE and CCME. Both extracts retain the anticancer effect on HSC-2 cell line. Thus, further studies should focus on the leaves of Cosmos caudatus.
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Keywords: Cosmos caudatus (Ulam raja), GC-MS analysis, Methanolic extraction, Aqueous extraction, Anti-OSCC

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) represents approximately 90% of all oral cancer cases making it the most prevalent and aggressive form of oral malignancy (1). Despite advancements in treatment modalities, OSCC continues to exhibit high morbidity and mortality rates, highlighting the urgent need for novel therapeutic strategies (2). The search for effective anticancer agents has increasingly turned towards natural products due to their diverse bioactive compounds and relatively low toxicity (3).

Cosmos caudatus (CC) commonly known as Ulam Raja in Malaysia (Fig. 1), is a plant endemic to Mesoamerica and belongs to the Asteraceae family (4). Traditionally, CC is used in Malaysian cuisine and as an herbal remedy to promote bone health, improve blood circulation, and alleviate halitosis (5). Recent studies have revealed a wide range of pharmacological activities attributed to CC, including antioxidant, anticancer, antihypertensive, antidiabetic, and antihyperlipidemic effects (6). These beneficial properties are linked to its rich phytochemical content, which includes flavonoids, phenylpropanoids, carotenoids, phenolic acids, sesquiterpene lactones, and vitamins (7).

A B

Fig. 1 : Shows the whole plant (A) and the leaf (B) of Cosmos caudatus

Previous research has reported the anticancer potential of CC against various cancer cell lines, such as HeLa (cervical cancer), T47D (breast cancer), and L1210 (leukemia) cells (8, 9). However, its effects on OSCC have not been explored. Given the complex nature of OSCC and the pressing need for new treatment options, investigating the cytotoxic effects of CC on OSCC cells could provide valuable insights into its therapeutic potential.

This study aims to evaluate the cytotoxicity of aqueous (CCAE) and methanolic (CCME) extracts of CC leaves on the human squamous carcinoma cell line (HSC-2). Additionally, we aim to identify the bioactive constituents responsible for the observed cytotoxic effects using gas chromatography-mass spectrometry (GC-MS) analysis. By understanding the specific compounds involved and their mechanisms of action, we hope to elucidate the potential of CC as a novel anticancer agent for OSCC.

MATERIALS AND METHODS

Plant processing

Whole CC plant was purchased from the regional area of Kota Bharu, Kelantan, Malaysia and authenticated by a botanist (voucher number: PID 441019-22). The leaves of CC were handily picked and washed thoroughly tap water followed by distilled water, then dried in an oven at 50°C. The dried leaves were weighed and ground using a blender (Panasonic®, Malaysia). The methanolic extraction was done according to Faujan et al. and the aqueous extraction was done according to Azwanida et al. with modification. The CC leaves powders were soaked in methanol at a ratio of 6:1 (methanol: grounded leaves) and in distilled water at 10:1 (distilled water: grounded leaves) (10, 11). The mixture was then placed on a reciprocating shaker (Stuart SSL2) at 200 strokes/min for 2 days. After shaking, the mixture was centrifuged at 25,000 rpm for 5 minutes (Primo R Centrifuges, Thermo Scientific), filtrated through Whatman filter paper No. 1, and the filtrate was collected in 50 ml falcon tubes. The extracts were concentrated using a rotary evaporator (Concentrator plus Eppendorf V-AL), frozen at -20°C, and subsequently freeze-dried using a freeze dryer (ScanVac CoolSafe). The dry powder of extract was stored at -20°C in 15 ml Falcon tube until further use.

Gas chromatography-mass spectrometry analysis

The chemical constituents of the CC extracts were analyzed using a Gas Chromatograph-Mass Selective Detector system (Agilent Technologies USA) model GC:7890A, MS: 5975C equipped with an HP-5 column (30 m length x 0.25 mm diameter x 0.25 µm film thickness). Helium (99.995% purity) was used as the carrier gas at a flow rate of 1.0 ml/min. The oven temperature was initially set at 50° C for 5 minutes, then increased to 300° C at a rate of 25°C/min and held for ten minutes. An auto-injector (7693 Autosampler, Agilent Technologies, USA) injected 1µl of the extract. The mass spectrometer scanned the samples in the range of m/z 40-650, with the electron ionization voltage set at 70eV. The quantity of each chemical compound was expressed as a percentage based on the peak area obtained from the chromatogram.

Identification of extract constituents

The identification of chemical constituents was based on mass spectral matching with NIST and WILEY libraries, with a match threshold of ≥ 80%.

Cell culture and MTT assay

Human squamous cell carcinoma (HSC-2) cells were purchased from RIKEN cell bank (Japan) (resource number: RBRC-RCB1945). Cells were cultured in complete minimum essential media (MEM) supplemented with 10% fetal bovine serum and 0.5% penicillin-streptomycin (Gibco™) under humid conditions at 37°C and 5% CO2. The cells were trypsinized and seeded into 96-well plate at a density of 5 Ч 105 cells/cm3 and allowed to adhere overnight. The following day, the cells were treated with CC methanolic extract (CCME) and CC aqueous extract (CCAE) at concentrations ranging from 6.25 to 0.0976 mg/ml, serially diluted in complete media, for 24, 48, and 72 hours. Before the cell viability assay, media-containing treatment was removed, and cells were washed with phosphate buffer saline (PBS) for 5 minutes on a shaker. After removing PBS, 100µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) diluted in plain media was added to each well, and the plates were incubated for four hours in the incubator. Subsequently, the MTT solution was removed, and 100µl of dimethyl sulfoxide (DMSO) was added to dissolve the formed formazan crystals. The absorbance was read using ELISA reader at a wavelength of 570 nm, with 600 nm as the reference wavelength.

Statistical analysis

All relative statistical analysis was conducted using GraphPad prism version 8.4.3 (686).

RESULTS

Batch Percentage Yield

Each batch (weighing 1kg) of CC leaves produced 20g of dried powder. The mean yield for the aqueous extract was 1.84% (standard deviation, SD = 2.96), and for the methanol extract, it was 1.39% (SD = 1.31). The highest yield was obtained from the aqueous extract of CC.

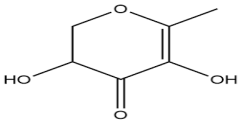
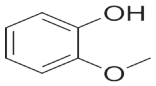
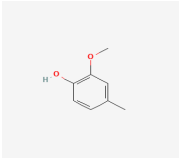
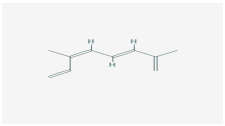
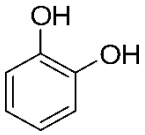
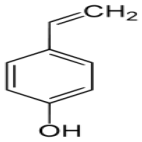
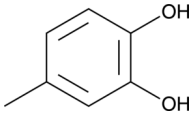
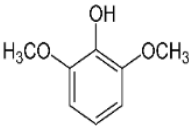
Physical Properties of the Extracts

The aqueous and methanolic extracts of CC exhibited similar physical properties. Both extracts were brown, odourless, and had a powdery texture.

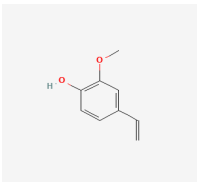
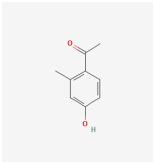
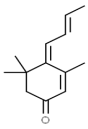
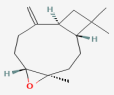
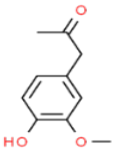
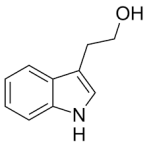
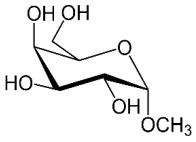
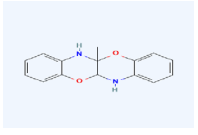
Bioactive Compounds of CC Extracts

Table 1 presents the bioactive compounds identified in the aqueous and methanolic extracts of CC. The mass spectrum and chromatography of these extracts are shown in Fig. 2 (A and B).

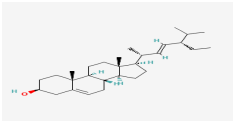
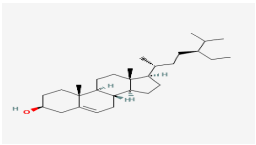
Table I : GC-MS profiled compounds of CCAE and CCME.

Compound	CCAE		CCME		C.F	M.W	Chemical structure	Library
	R.T.	Total %	R.T.	Total %				
2,3-Dihydro-5-hydroxy-6-methyl-4H-pyran-4-one	N.D.	N.D.	7.85	0.25	C_6H_8O	128.12		Chembook
Guaiacol	7.88	1.30	7.88	0.56	$C_7H_8O_2$	124.13		PubChem
Creosol	8.71	0.11	N.D.	N.D.	$C_8H_{10}O_2$	138.16		PubChem
2,6-Dimethyl-1,3,5,7-octatetraene, E,E-	N.D.	N.D.	8.21	0.79	$C_{10}H_{14}$	134.21		PubChem
Catechol	8.83	11.49	8.83 8.98	2.87 1.02	$C_6H_6O_2$	110.1		PubChem
4-vinyl-phenol	8.89	4.66	8.9	4.27	C_8H_8O	120.15		PubChem
Homocatechol	9.20	0.83	9.23 9.45	0.42 1.27	$C_7H_8O_2$	124.13		PubChem
Syringol	N.D.	N.D.	9.71	1.4	$C_8H_{10}O_3$	154.16		PubChem

CONTINUE

Compound	CCAE		CCME		C.F	M.W	Chemical structure	Library
	R.T.	Total %	R.T.	Total %				
2-Methoxy-4-vinyl-phenol	9.44 9.49	2.70 2.65	N.D.	N.D.	$C_9H_{10}O_2$	150.17		PubChem
4-Hydroxy-2-methylac-etophenone	N.D.	N.D.	9.49	2.75	$C_9H_{10}O_2$	150.17		PubChem
Megastigmatrienone A	N.D.	N.D.	9.49	2.75	$C_{13}H_{18}O$	190.28		ChemSpider
Indole	N.D.	N.D.	9.39	0.15	C_8H_7N	117.15		PubChem
Caryophyllene oxide	N.D.	N.D.	10.99	0.25	$C_{15}H_{24}O$	220.35		PubChem
Guaiacyl acetone	N.D.	N.D.	10.69	3.12	$C_{10}H_{12}O_3$	180.20		PubChem
Tryptophol	10.76	0.52	N.D.	N.D.	$C_{10}H_{11}NO$	161.20		PubChem
Methyl dodecanoate	10.58	0.08	N.D.	N.D.	$C_{13}H_{26}O_2$	214.34		PubChem
Methyl α-D-galactoside	N.D.	N.D.	11.25	2.61	$C_7H_{14}O_6$	194.18		PubChem
Cyclopropaneoctanoic acid, 2-hexyl-,methyl ester	12.70	0.63	N.D.	N.D.	$C_{18}H_{34}O_2$	310.5		Wiley
[1,4]Benzoxazino [3,2-b][1,4]benzoxazine, 5a,6,11a,12-tetrahy-dro-5a-methyl-	12.95 13.95	0.50 0.36	N.D.	N.D.	$C_{15}H_{14}N_2O_2$	254.28		PubChem

CONTINUE

Compound	CCAE		CCME		C.F	M.W	Chemical structure	Library
	R.T.	Total %	R.T.	Total %				
Stigmasterol	17.65	0.22	N.D.	N.D.	$C_{29}H_{48}O$	412.70		PubChem
γ-Sitosterol	18.05	0.11	N.D	N.D	$C_{29}H_{52}O_2$	414.7		PubChem

M.W.= molecular weight (g/mol).
C.F.= chemical formula
R.T.=retention time (min).
N.D. = not detected.

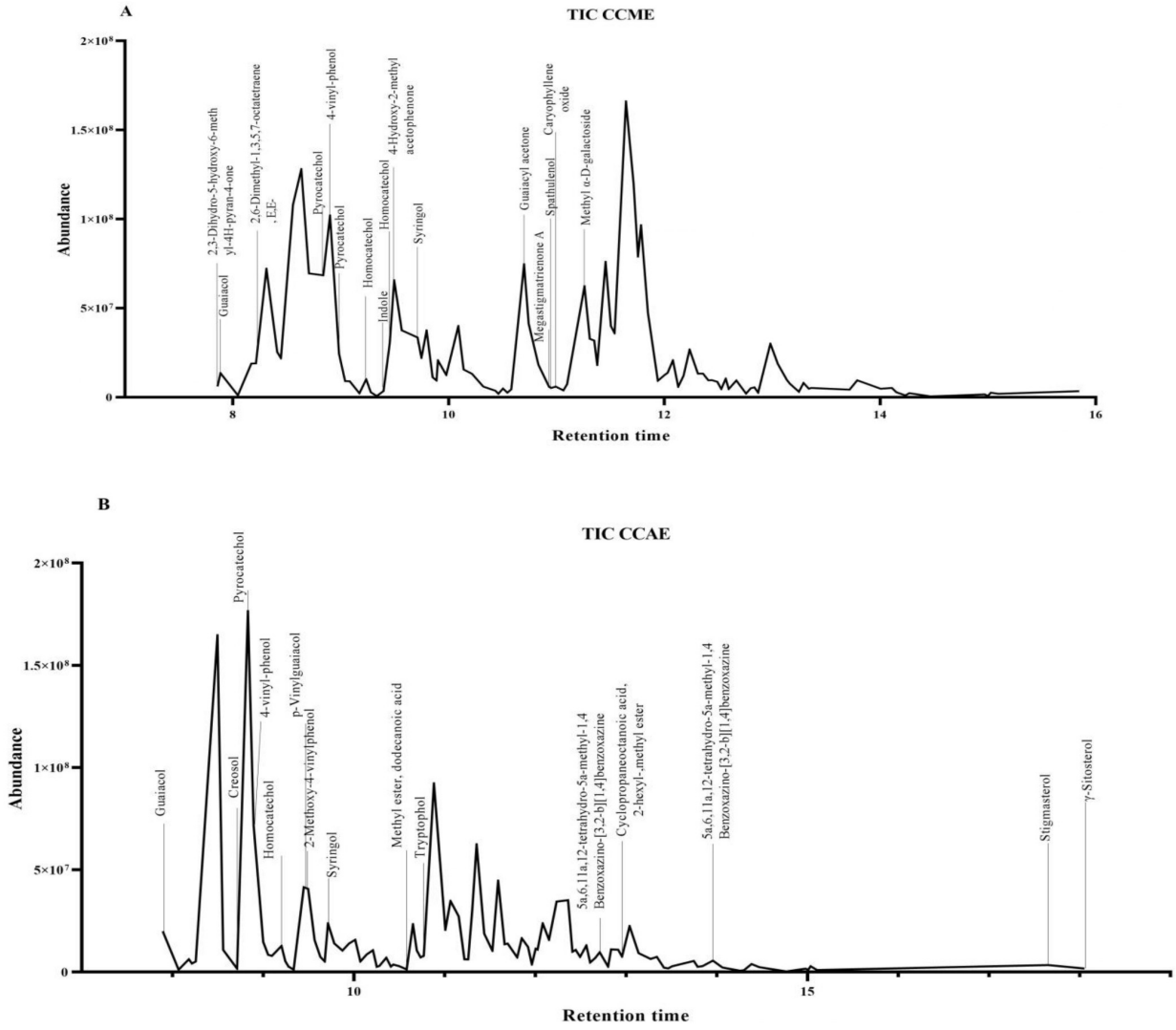


Fig. 2 : Total ionic chromatogram (TIC) of (A) CCME (B) CCAE.

The major phytochemical groups recovered from the extracts were phenols, accounting for 25.29% in the CCAE and 17.68% in the CCME. Alkaloids were presented in low percentages, with 0.15% in the methanol extract and 0.52% in the aqueous extract. Terpenoids were detected only in the methanol extract, constituting 1.51%. Steroids were found exclusively in the aqueous extracts (Table 1).

Cytotoxicity of CC Extracts on HSC-2 cell line

Table 2 shows the IC50 of CCAE and CCME. Both extracts exhibited a cytotoxic effect on HSC-2 cell line, with CCME showing a greater cytotoxic effect than CCAE. Conversely, CCME showed a consistent trend across all three-time points, significantly inhibiting HSC-2 cells (Fig. 3).

Table II : IC_{50} of CCAE and CCME.

Timeline (hours)	Extract	IC_{50} (mg/ml)	95% CI	R^2
24	CCAE	N.D	-	-
	CCME	0.26±0.45	0.132-0.539	0.7954
48	CCAE	2.1±3.63	1.284-3.790	0.9403
	CCME	0.24±0.41	0.101-0.621	0.6512
72	CCAE	0.93±1.61	0.509-1.79	0.8804
	CCME	0.31±0.53	0.125-0.912	0.6005

*N.D. = not detected
CI = confidence interval

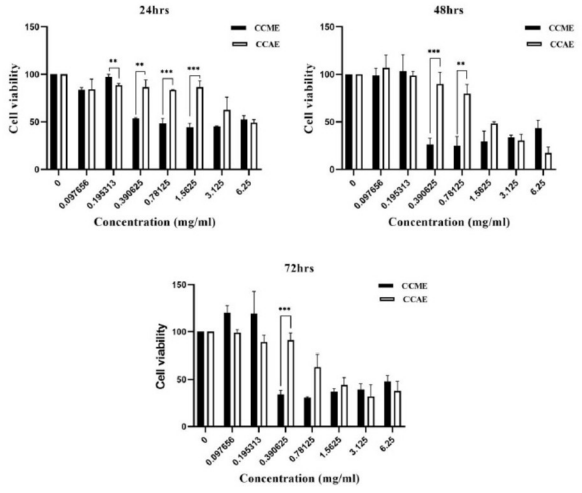


Fig. 3 : The barograph shows the cytotoxic effects of CCAE and CCME on HSC-2 cells at 24, 48, and 72 hours. ** significant at P<0.002, * significant at P<0.001.**

DISCUSSION

Cosmos caudatus is known for its diverse medicinal properties, which are attributed to the presence of various phytochemical agents. Previous studies have reported its pharmacological activities, including antimicrobial and antioxidation effects. Additionally, the cytotoxicity of CC extends beyond microorganisms to cancer cells, with documented effects on cervical, breast, and leukemia cancers.

The extraction yield reported in this study approximately falls within the guidelines of Kooperation Phytopharmaka (2016), in which the average ratio of a plant material to extract produced is 10:1 (12). The extraction yield reported in this study was approximately 8-9%. In comparison to other studies, the yield was relatively higher than those reported by Faujan et al. and Rameli et al. (1.53%, and 1.13%, respectively) (13), and lower than those who reported the use of similar extraction solvents but with the application of external factors viz. sonication and heat which allows solvent penetration and increase the extraction yield significantly (14).

Our GC-MS analysis identified four major phytochemical groups: phenols, terpenoids, alkaloids, and steroids. Phenols were the most abundant, particularly in the CCAE, followed by terpenoids which were exclusively present in CCME. This finding aligns with previous analyses by (15). Alkaloids were detected in both extracts, consistent with a previously published report (16). Traces of steroids were found only in CCAE (Table 1). Furthermore, our analysis revealed a total of 13 bioactive compounds, with 10 found in CCME and 9 in CCAE. Both extracts shared common compounds, including, guaiacol, pyrocatechol, homocatechol, and syringol (Table 1).

The use of gas chromatography as a mobile phase enabled a broader profiling of CC extract compared to previous studies using liquid chromatography. For instance, Firdaus et al. identified only 2 compounds (Genistin and Gentiatibetine) in CCAE using LCMS/MS (17). While Mediani et al. profiled four bioactive compounds (quercetin rhamnoside, quercetin glucoside, rutin, chlorogenic acid) in CCME using HPLC and Nuclear magnetic resonance (NMR) (18). Rafi et al. applied LC-HRMS on CC leaves extract and reported the presence of quercitrin, avicularin, isoquercitrin, quercetin-3-O-rutinoside, vitexin, and rutin (7)

The detected phenols possess various biological activities. For instance, Pyrocatechols (Catechols) are excellent regulators of metabolism and regular physiological activities and used in anti-parkinsonian, adrenergic, anti-hypertensive, and bronchodilator drugs (19). p-Vinylguaiacol has variety of activities such as antimicrobial, anti-inflammatory, fungicide, and

antioxidant (20). Syringol and guaiacol have antioxidant and radical scavenging activities, and the latter retains anti-fungal effects (21), especially on Candida species (22). Creosol has been reported to exhibit anti-microbial, antioxidant, and anti-tumour effects (23). Alkaloids procured from CC extracts (indole and tryptophol) retain important biological activities. Indole with its ring system 1, is considered a crucial structural moiety in drug development. Drugs such as vincristine, and vinblastine (anti-tumor) are Indole alkaloid derivatives (24). Hamid et al. (2017) proposed employing indole as a natural anti-depressant. Tryptophol possesses antifungal activity (25). Terpenoids i.e. spathulenol and caryophyllene oxide possess antibacterial and antifungal properties (26). Caryophyllene oxide retains analgesic and anticancer effects (27). Steroids were only present in CCAE. γ–Sitosterol retains an anti-diabetic effect and is used to treat maladies like ulcers, bronchitis, and cardiovascular diseases (28).

The cell viability assay demonstrated that both extracts exerted a cytotoxic effect on the HSC-2 cell line (Fig. 3). CCAE yielded higher percentages of these compounds, yet it showed weaker cytotoxicity compared to CCME. Other constituents found in CCME that are not detected with GC-MS analysis might be associated with anti-HSC-2 activity like β-carotene and ascorbic acid (15, 29). Moreover, CCME has been noted for its potent activity against breast and leukemia cancers (30). Previous studies have highlighted the superior pharmacological activity of CCME over CCAE in terms of radical scavenging and anti-inflammatory (15, 31). The anticancer effect of CC is reported to work on the apoptotic genes. Sandra et al. (2024) reported an increase in Bax protein and a decrease in Bcl-2 protein content of oral cancer cells (HSC-3). They also reported the release of cytochrome C from the mitochondria of HSC-3. This activity is likely to be caused by quercetin (a catechol moiety) (32). Another possible anticancer target of CC leaf extract is the extracellular signal-related kinase (ERK2) due to the presence of pyrocatechol which is a compound reported to inhibit the human H460 and murine KP2 lung cancer cell lines by targeting ERK2 kinase (33). Other profiled bioactive compounds in this study are P-Vinylguaiacol and stigmasterol. P-Vinylguaiacol was reported to inhibit HT-29 and HCT-116 (colon cancer) cell lines by inducing cell cycle arrest at the G1 phase (34). Stigmasterol showed promising anticancer effects on the liver, ovarian, lung, and breast by targeting multiple cellular mechanisms. It can regulate PI3K/Akt signalling pathway, triggering cellular apoptosis, generating reactive oxygen species (ROS) in the mitochondria of cancer cells, and affecting cyclin-dependent kinase (CDK) proteins. Its anti-proliferative activity is mainly dependent on its modulatory effect on cyclin proteins and cyclin-dependent kinase (CDK) (35).

This study reported promising anti-oral cancer activity of CC leaf extract. Although the extract showed promising cytotoxic effects, the exact mechanisms underlying its activity against OSCC cells remain unclear. Further studies are needed to explore pathways such as apoptosis induction, cell cycle arrest, and oxidative stress modulation. The cytotoxic evaluation was limited to a single OSCC cell line. Testing the extract on additional cancer cell lines and normal oral cells would provide a broader understanding of its selectivity and efficacy.

CONCLUSION

Both methanolic and aqueous extracts of CC leaves yielded phenols and alkaloids, with aqueous extracts showing higher percentages of these compounds compared to methanolic extracts. Distinct differences were noted, with steroids detected exclusively in aqueous extracts and terpenoids only in methanolic extracts. Importantly, both extracts showed cytotoxic effect on OSCC (HSC-2) cell line due to the presence of significant bioactive compounds known for their antioxidant, antimicrobial, antifungal, and anticancer properties.

Future studies should optimize extraction method to improve the extraction yield, alternative techniques such as ultrasonic-assisted extraction, supercritical fluid extraction, or solvent optimization should be explored to ensure higher recovery of bioactive compounds. Employment of advanced analytical techniques, such as NMR or HPLC can provide comprehensive metabolite profiling for CC extract to identify and characterize non-volatile bioactive metabolites that might have been missed by GC-MS. In addition, investigation for the molecular pathways underlying the cytotoxic effects of the extract on OSCC cells, such as apoptosis, oxidative stress, and autophagy, to better understand its mechanism of action.

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