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Research Article
Applications of Chromatographic Techniques for Fingerprinting of Toxic and Non-toxic *Euphorbia* Species

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Abstract

Background and Objective: *Euphorbia* species have historically been used as medicinal plants to treat different ailments. However, some species have been reported to exhibit various degrees of toxicity. It becomes critical to distinguish toxic species from those that are non-toxic, for a particular application. The aim of the study was to determine the method for fingerprinting the chemical constituents of the selected toxic and non-toxic *Euphorbia* species to identify markers of toxicity. **Material and Methods:** Hexane, DCM, methanol, ethyl acetate and water plant extracts of *Euphorbia ammak*, *clavarioides*, *caerulescens*, *polygona* and *trigona* were investigated for their cytotoxic activities towards the mammalian Vero cell line using MTT cell viability test assay. The presence of secondary metabolites and proteins were assessed in the plant extracts. Moreover, the study used chromatographic methods to fingerprint the plant extracts to identify toxicity markers. **Results:** The DCM extract of *E. ammak* exhibited the highest cell growth inhibition at all concentrations tested. The non-polar extracts of *E. clavarioides* exhibited the highest cell growth inhibition activity with hexane extract reaching IC_{50} at 1 μg mL^{-1}. The DCM extract of *E. caerulescens* reached IC_{50} at a concentration of 10 μg mL^{-1}, while other extracts didn't show any activity. The hexane and DCM extracts of *E. polygona* exhibited the highest cell growth inhibition activity, reaching IC_{50} at a concentration of 10 μg mL^{-1}. All 4 extracts of *E. trigona* didn't show cell growth inhibition. All *Euphorbia* species showed the presence of secondary metabolites. The biuret and xanthoprotein methods indicated that there were no proteins detected in all 5 *Euphorbia* species. TLC profiles of toxic extracts revealed additional bands which were absent in non-toxic species. **Conclusion:** It is concluded that the TLC method developed in this study can be used as a quick screen method to possibly distinguish toxic from non-toxic species, as well as in identifying the studied species.

Key words: *Euphorbia ammak*, *clavarioides*, *caerulescens*, *polygona*, *trigona*, phytochemical analysis, cytotoxicity, protein detection, TLC profiling, Vero cell line

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The use of analytic chromatographic techniques for fingerprinting of plant species has brought solutions to challenges of species identification, qualitative and quantitative analysis of plants' constituents as well as standardization of plant-based health products. There is still widespread preference to use medicinal plants in their natural form as they are claimed to be safer, with less side effects compared to pharmaceutical drugs[1,2]. However, the inability to correctly identify, distinguish similar species and ensure desired constituents are present at the effective levels, is a challenge facing development of plant-based medicines. Lack of standardization of plant extracts affects the efficacy of treatments and consistency of treatment outcomes. Proper identification of plants through chemical fingerprinting gives a library that can be used to identify plant species and predict effectiveness by assessing the quality and quantity of identified active constituents[3].

Euphorbia is a very diverse genus of flowering plants that belong to the family *Euphorbiaceae*[4]. Most species from the genus *Euphorbia* typically produce a white milky sap called latex which has been reported to have some degree of toxicity[5-7]. This latex is relatively irritating to those who are allergic to it. However, it is quite useful to the plants themselves as defence mechanism[8,9]. The latex and the aerial parts of *Euphorbia* species have, historically, been used to treat different ailments such as wounds, warts and headaches[10,11]. The genus has some species that have pharmacological properties such as antimicrobial, anti-fungal, antiviral and anticancer properties[5]. The white latex is also reported to be used in various applications other than medicine e.g., production of rubber, reduction in nanotechnology etc[9]. It becomes critical therefore to distinguish the toxic species from non-toxic species in order to categorize the species for different applications and uses. It is therefore useful that there be a chemical fingerprint of these species to identify markers of toxicity.

Euphorbia plants have been reported to comprise of various constituents such as steroids, phenolics, cerebrosides, glycerols, flavonoids, glycosides, tannins, saponins, alkaloids, pentose, anthraquinones, phytosterols, terpenes including; diterpenes and triterpenes[9,12,13]. They have also been reported to contain biologically active proteins such as proteases, chitinases, oxidases and lectins. A combination of phytochemical constituents and proteins implies different extraction and identification methods for these species of plants.

The study aimed to determine the method for fingerprinting the chemical constituents of the selected *Euphorbia* species (*ammak*, *clavarioides*, *caerulescens*, *polygona* and *trigona*) to identify common markers of toxicity. The chemical fingerprinting will assist in quick screening of *Euphorbia* species to determine whether the species is toxic or not and to help determine whether the tested species contains the necessary chemical composition for the intended application. The elucidation of the chemical structures of the compounds depicted by bands/peaks on the chromatograms falls outside of the scope of this study.

MATERIALS AND METHODS

Plant collection and extraction: The study was conducted at the Central University of Technology, Health and Environmental Sciences and University of the Free State, Pharmacology, Bloemfontein, South Africa between July, 2018 and September, 2019. Five species of *Euphorbia* were collected from Lesotho and KwaZulu-Natal Province of South Africa, between February-April, 2019. Plants were authenticated by a Botanist at University of the Free State. Fresh plants were chopped into small pieces, left to dry at room temperature and ground to fine powder. Crude extract was obtained by homogenizing 10 g of powdered material with 100 mL of distilled water and sequentially with organic solvents in their increasing order of polarity starting with hexane, dichloromethane, ethyl acetate and methanol. Another 10 g of plant material was extracted with 100 mL of acetone.

Plant samples were soaked in the solvent for 48 h on a shaker (FMH instruments, sepsci), then filtered with a filter paper (whatman® Maidstone). Organic filtrates were dried by rotary evaporation (Buchi, labotech Switzerland) at 45°C, placed under fume hood until dry. Water extracts were concentrated by Freeze drying. Dried extracts were stored at 4°C until further use.

The percentage yield was calculated using the formula:

$$\text{Yield (\%)} = \frac{\text{Concentrated plant extract}}{\text{Dried plant material}} \times 100$$

Phytochemical screening: The whole ground plant materials of *Euphorbia ammak*, *clavarioides*, *caerulescens*, *polygona* and *trigona* were screened for phytosterols, pentose,

tannins, glycosides, triterpenoids, anthraquinones, saponins, flavonoids and alkaloids based on the protocols by Bhandary *et al.*[14] and Yusuf *et al.*[15].

Cytotoxicity screening: The mammalian Vero cell line was obtained from cellonex, South Africa. Cells were cultured in complete medium, DMEM supplemented with 10% fetal bovine serum (FBS) and maintained in an incubator (NUVE EC 160) at 37°C, 5% CO_2. Cells were sub-cultured when 90% confluent, by trypsinization. Cells were centrifuged at 800 rpm for 5 min to obtain a pellet. Cells were re-suspended in 5 mL of the medium. Viability of the cells was assessed using trypan crystal blue dye and cells were counted using automated cell counter (countess FL, life technologies). The cells were seeded at density of 1×10^5 cells mL^{-1} in 96 well plates, followed by incubation for 24 h at 37°C temperature. Following incubation, cells were treated with 100 µL of test extracts added in triplicates. The stock solutions of the test samples (20 mg mL^{-1}) were prepared in DMSO, diluted to concentrations of 100, 10 and 1 µg mL^{-1} in complete medium. Emetine was used as control standard drug. The plates were then incubated for further 48 h. Cell viability was measured using the MTT assay and absorbance was read at 540 nm wavelength. Results were analyzed using Microsoft excel.

Protein detection: Methanol extracts of all five plants were dissolved and prepared in warm distilled water. The extracts were tested for detection of proteins using biuret and xanthoprotein tests. For Biuret test, sodium hydroxide (NaOH) and a few drops of copper sulfate ($CuSO_4$) solutions were added to the sample solution. A violet or pink colour was observed as indicated in Fig. 6. For Xanthoprotein test, concentrated sulfuric acid (H_2S0_4) was added to the sample solution. A white precipitate was formed as indicated in Fig. 7. In both tests, wheat flour was used as positive control.

Plant extract fingerprinting by TLC: The study used thin layer chromatography to fingerprint the plant extracts of *Euphorbia* species. Silica gel on thin aluminium plates (5×10 cm) was used as stationary phase. For mobile phase, 3 different solvent systems: Toluene -acetone (8:2) (non -polar solvent), Toluene-chloroform-acetone (40:25:35) (Semi-polar solvent) and n-butanol-glacial acetic acid-water (50:10:40) (Polar solvent) were used in elution. Dried extracts were reconstituted (2 mg mL^{-1}) in the solvent used for extraction and spotted on the TLC plates using a capillary tube. The plates were developed in the appropriate mobile system then visualised under ultraviolet (UV) light. The Rf values were determined and used to compare the chemical profile of the samples and to identify the presence/absence of toxicity markers in different plant species of *Euphorbia*. The intensity of the bands on TLC plates was a qualitative measure of the level of phytochemicals in each plant.

Statistical analysis: The values are presented as the mean±standard deviation (SD).

RESULTS

The percentage yield of the dried plant extracts was calculated and results are summarised in Table 1. Generally, water and methanol had the highest yields in all plants extracted.

All 5 *Euphorbia* species confirmed the presence of phytosterols, glycosides, triterpenoids, flavonoids and alkaloids. Anthraquinones were detected in three *Euphorbias ammak*, *clavarioides* and *trigona*. Tannins were detected only in *Euphorbias ammak*, *caerulescens* and *polygona*. Pentose and saponins were not present (Table 2).

Cytotoxicity screening: The graphs in Fig. 1-5 comparatively show the cell growth inhibition of extracts of *Euphorbia ammak*, *clavarioides*, *caerulescens*, *polygona and trigona.* Growth inhibition of 50% of the cell culture (IC_{50} value) is marked on each graph and used as an indicator for activity. Extracts that showed 50% growth inhibition at 10 µg mL^{-1} and below were considered active.

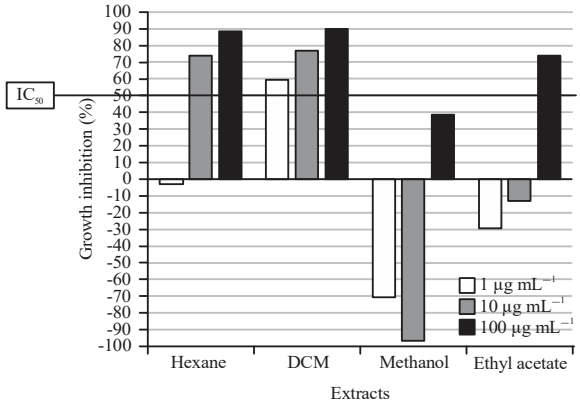

Fig. 1: Effects of *Euphorbia ammak* extracts on Vero cell growth inhibition
DCM extract achieved more than 50% inhibition at all concentrations tested

Table 1: Percentage yields of 5 *Euphorbia* species following extraction with different solvents

	Plant samples				
	Yield/solvent (%)				
Plant names	Hexane	DCM	MeOH	EtOAc	H_2O
Euphorbia ammak (whole plant)	5.60	1.70	6.30	0.10	14.54
Euphorbia clavarioides (whole plant)	4.10	2.00	9.10	0.30	20.40
Euphorbia caerulescens (whole plant)	2.40	3.00	7.60	0.80	11.70
Euphorbia polygona (whole plant)	2.14	2.76	4.39	0.61	4.40
Euphorbia trigona (whole plant)	1.99	1.49	12.29	0.89	8.10

Table 2: Phytochemical Screening of ground powdered material of *Euphorbia* plants

	Euphorbia species				
Phytochemicals	*Euphorbia ammak*	*Euphorbia clavarioides*	*Euphorbia caerulescens*	*Euphorbia polygona*	*Euphorbia trigona*
Phytosterols	+	+	+	+	+
Pentose	-	-	-	-	-
Tannins	+	-	+	+	-
Glycosides	+	+	+	+	+
Triterpenoids	+	+	+	+	+
Anthraquinones	+	+	-	-	+
Saponins	-	-	-	-	-
Flavonoids	+	+	+	+	+
Alkaloids	+	+	+	+	+

+: Present, -: Not detected

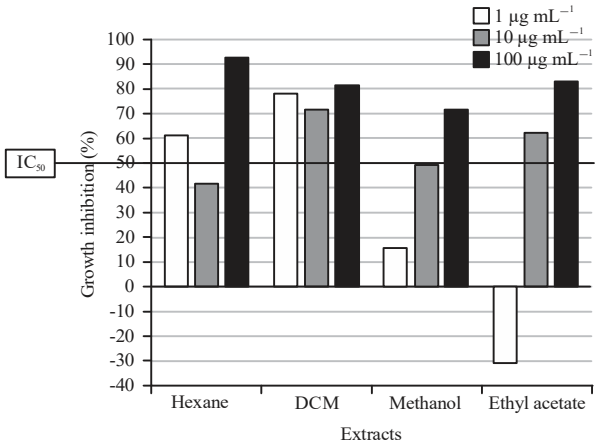

Fig. 2: Effects of *Euphorbia clavarioides* extracts on Vero cell growth inhibition

All extracts reached IC_{50} at concentrations of 100 µg mL^{-1}, Hexane and DCM extracts achieved an IC_{50} even at 1 µg mL^{-1}

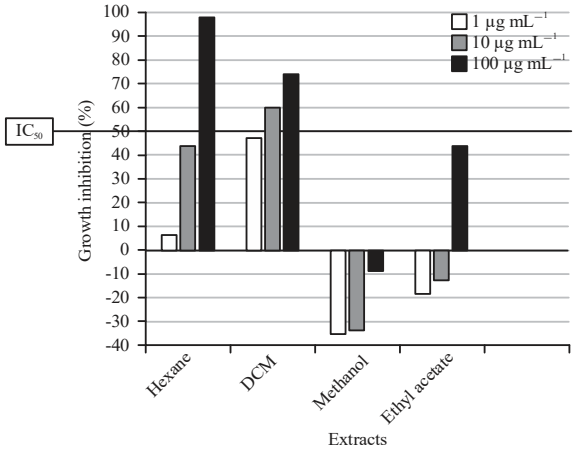

Fig. 3: Effects of *Euphorbia caerulescens* extracts on Vero cell growth inhibition

The DCM extract of *E. ammak* exhibited the highest cell growth inhibition at all 3 concentrations, while the hexane extract showed activity at concentrations of 10 and 100 µg mL^{-1}. Methanol and ethyl acetate extracts did not show any activity. Additionally, proliferation of Vero cells was observed at concentrations of 1 and 10 µg mL^{-1} for methanol and ethyl acetate extracts (Fig. 1).

All four extracts of *E. clavarioides* exhibited cell growth inhibition activity, although decreased activity was observed in methanol and ethyl acetate at a concentration of 1 µg mL^{-1}. Additionally, proliferation of Vero cells was observed at a minimum concentration (1 µg mL^{-1}) for ethyl acetate extracts (Fig. 2). This plant can be regarded as cytotoxic.

Three extracts (hexane, methanol and ethyl acetate) of *E. caerulescens* were not active against the Vero cell line. There was inhibition only at concentrations of 10 and 100 µg mL^{-1} for DCM extract. Additionally, proliferation of Vero cells was observed for methanol and ethyl acetate (1 and 10 µg mL^{-1}) extracts (Fig. 3).

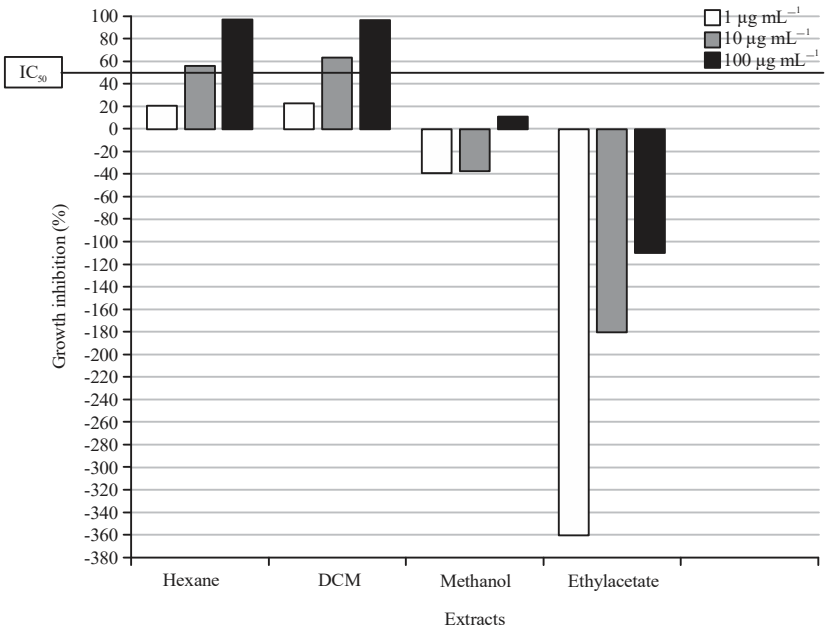

Fig. 4: Effects of *Euphorbia polygona* extracts on Vero cell growth inhibition

Table 3: Protein detection results of *Euphorbia ammak*, *clavarioides*, *caerulescens*, *polygona* and *trigona*, the whole plant was tested

Species	Xanthoprotein test	Biuret test
Euphorbia ammak		
Euphorbia clavarioides		
Euphorbia caerulescens		
Euphorbia polygona		
Euphorbia trigona		

Hexane and DCM extracts of *E. polygona* exhibited cell growth inhibition activity at concentrations 10 and 100 µg mL^{-1}. The methanol and ethyl acetate extracts didn't show any activity at all 3 concentrations. It can be deduced that the cytotoxic molecules in this plant are non-polar, since only Hexane and DCM extracts were active. Proliferation was observed in polar extracts (Fig. 4).

All 4 extracts of *E. trigona* did not inhibit 50% of the cell growth at 10 µg mL^{-1} and below, and therefore were not considered cytotoxic (Fig. 5).

Protein detection: *Euphorbia* plants have been reported to contain biologically active proteins[16]. Methanol extracts were subjected to detection of proteins using biuret and xanthoprotein tests. Positive control results are shown in Fig. 6 and 7. Figures in Table 3 show protein detection results for *Euphorbia ammak*, *clavarioides*, *caerulescens*, *polygona* and *trigona*. In all the methanol extracts of the studied *Euphorbias*, proteins were not detected using both tests.

Thin layer chromatography: After separation of the extracts, the TLC profiles of toxic extracts revealed additional bands which were absent in non-toxic species (Fig. 8).

TLC profiling results of hexane extracts showed that *E. polygona* had the highest number of bands, followed by *Euphorbia ammak* and *caerulescens* with 4 bands, *clavarioides* with 2 bands and *trigona* with no bands (Table 4).

TLC profiling results of DCM extracts showed that *E. caerulescens* had the highest number of bands, followed by *Euphorbia polygona* with 9 bands, *ammak* with 8 bands, *trigona* with 7 bands and *clavarioides* with 6 bands (Table 5).

Table 4: TLC profiling results of hexane extracts

Samples	Number of bands	Rf values
Euphorbia ammak	4	0.64, 0.65, 0.66, 0.95
Euphorbia clavarioides	2	0.77, 0.79
Euphorbia caerulescens	4	0.66, 0.67, 0.69, 0.97
Euphorbia polygona	5	0.62, 0.64, 0.79, 0.69, 0.97
Euphorbia trigona	0	0

Number of bands produced when visualized under UV light and the Rf values determined

Table 5: TLC profiling results of DCM extracts

Samples	Number of bands	Rf values
Euphorbia ammak	8	0.2, 0.32, 0.44, 0.45, 0.55, 0.72, 0.94, 0.96
Euphorbia clavarioides	6	0.44, 0.45, 0.5, 0.55, 0.94, 0.96
Euphorbia caerulescens	10	0.45, 0.48, 0.55, 0.72, 0.75, 0.77, 0.81,0.85, 0.94, 0.96
Euphorbia polygona	9	0.45, 0.55, 0.56, 0.72, 0.75, 0.77, 0.85, 0.93, 0.96
Euphorbia trigona	7	0.44, 0.45, 0.55, 0.72, 0.85, 0.94, 0.96

Number of bands produced when visualized under UV light and the Rf values determined

Table 6: TLC profiling results of methanol extracts

Samples	Number of bands	Rf values
Euphorbia ammak	3	0.62, 0.74, 0.90
Euphorbia clavarioides	2	0.86, 0.92
Euphorbia caerulescens	4	0.59, 0.71, 0.73, 0.90
Euphorbia polygona	4	0.58, 0.70, 0.73, 0.92
Euphorbia trigona	2	0.56, 0.73

Number of bands produced when visualized under UV light and the Rf values determined

Table 7: TLC profiling results of ethyl acetate extracts

Samples	Number of bands	Rf values
Euphorbia ammak	3	0.55, 0.65, 0.8
Euphorbia clavarioides	3	0.49, 0.63, 0.79
Euphorbia caerulescens	0	0
Euphorbia polygona	4	0.55, 0.65, 0.78, 0.81
Euphorbia trigona	3	0.55, 0.65, 0.81

Number of bands produced when visualized under UV light and the Rf values determined

Table 8: TLC profiling results of water extracts

Samples	Number of bands	Rf values
Euphorbia ammak	1	0.91
Euphorbia clavarioides	2	0.57, 0.92
Euphorbia caerulescens	2	0.57, 0.92
Euphorbia polygona	1	0.93
Euphorbia trigona	0	0

Number of bands produced when visualized under UV light and the Rf values determined

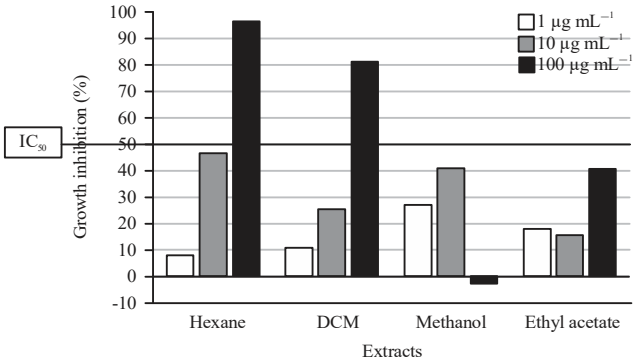

Fig. 5: Effects of *Euphorbia trigona* extracts on Vero cell growth inhibition
Hexane and DCM extracts reached IC_{50} only at the highest concentration tested (100 µg mL^{-1})

Fig. 6: Biuret test using flour

Fig. 7: Xanthoprotein test using flour

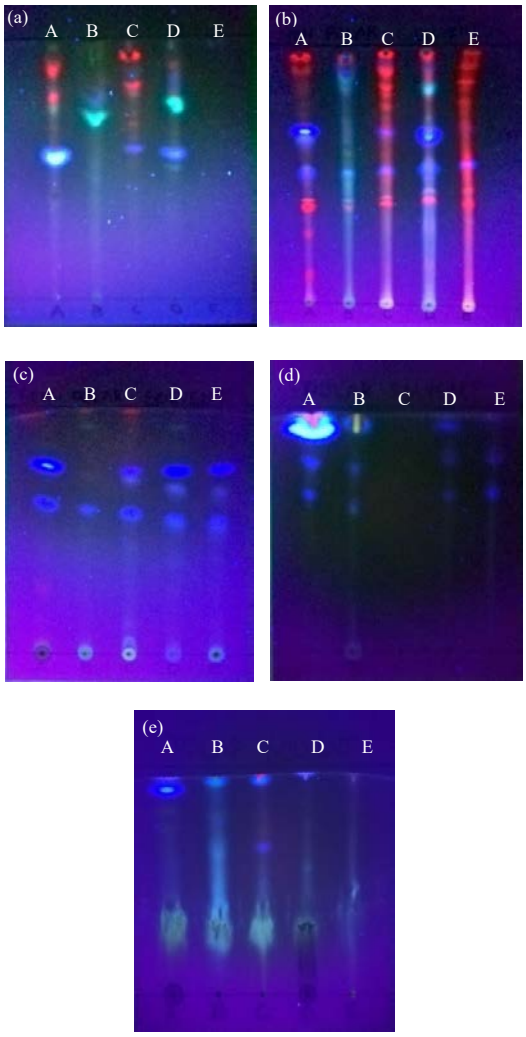

Fig. 8(a-e): TLC profiling results of (a) Hexane extracts, (b) DCM extracts, (c) Methanol extracts, (d) Ethyl acetate extracts and (e) Water extracts of *Euphorbia* species

A: *Euphorbia ammak*, B: *Euphorbia clavarioides*, C: *Euphorbia caerulescens*, D: *Euphorbia polygona*, E: *Euphorbia trigona*

TLC profiling results of methanol extracts showed that *Euphorbia caerulescens* and *polygona* had the highest number of bands, followed by *Euphorbia ammak* with 3 bands and *Euphorbia clavarioides* and *trigona* with 2 bands each (Table 6).

TLC profiling results of ethyl acetate extracts showed that *Euphorbia polygona* had the highest number of bands, followed by *Euphorbia ammak*, *clavarioides*, *trigona* with 3 bands. *Euphorbia caerulescens* had no bands (Table 7).

TLC profiling results of water extracts showed that *Euphorbia clavarioides* and *caerulescens* had the highest number of bands with 2 bands each, followed by *Euphorbia ammak* and *polygona* with 1 band each. *Euphorbia trigona* had no bands (Table 8).

DISCUSSION

In this paper, the study suggests classification of cytotoxic and non-cytotoxic species based on cytotoxicity screening, phytochemical screening and profiling.

Generally, the polar solvents, water and methanol had the highest percentage yields of the 5 *Euphorbia* species (Table 1). Phytochemical analysis c onfirmed the presence of phytosterols, glycosides, triterpenoids, anthraquinones, saponins, flavonoids and alkaloids for all 5 *Euphorbia* species.

Only *Euphorbia ammak*, *caerulescens* and *polygona* showed the presence of tannins. All 5 *Euphorbia* lacked pentose and saponins (Table 2).

Phytosterols have been used for the last half-century because of their cholesterol-lowering properties and other potential health benefits[17]. Tannins have been reported to cause regression of tumors that are already present in tissue, implying their potential in anti-proliferation of cancer cells activity[18-21]. Francisco and Pinotti[22] and Simin *et al.*[23] have reported that the existence of glycosides in some plant species often indicates toxicity. Chudzik *et al.*[24] have reported that triterpenoids have innumerable biological activities such as anti-cancer properties, not only they are responsible for growth inhibition of cell lines but also play a major role in inducing apoptosis of cancer cells.

Plant extracts showing the presence of anthraquinones are increasingly used for pharmaceuticals due to their therapeutic and pharmacological properties[25]. Flavonoids have a wide range of biochemical and pharmacological effects, including antioxidant, anti-inflammatory and antifungal effects[26]. Aniszewski[27] reported that one of the most common biological properties of alkaloids is their cytotoxicity. Their cytotoxicity has been studied against cancer cell lines. Aniszewski[27] regarded toxicity as a secondary function of alkaloids because they are generally non-toxic to the organisms producing them. The biotoxicity of alkaloids is selective and dependent on different organisms and the chemical structure of alkaloids themselves[27].

Secondary metabolites may not exert therapeutic effects or related bioactivities directly, but could enhance bioactivities of other components by acting synergistically, thereby modulating the effects of the plants[28]. The cytotoxicity of *Euphorbia ammak*, *clavarioides*, *caerulescens*, *polygona* and *trigona* could result from the presence of phytochemicals.

Secondary metabolites may be used as chemical markers for qualitative and quantitative assessment, while specific toxic components may be used as bioactive compounds in screening[28]. The results from this study suggest that the cytotoxic molecules in the studied *Euphorbia* plants are non-polar, since only the non-polar extracts showed activity while the more polar extracts were not active.

The extracts were tested for detection of proteins using biuret and xanthoprotein tests. For biuret test, a violet or pink colour was not observed as indicated in Table 3. For xanthroprotein test, a white precipitate was not formed as indicated in Table 3. It could be deduced that *Euphorbia ammak*, *clavarioides*, *caerulescens*, *polygona* and *trigona* don't have any proteins.

Species with the highest bands produced in the TLC profiles imply high amount of chemically varied phytochemicals (Fig. 8). Based on the results obtained, chemical components that are present in *Euphorbia* species may have more than one attribute and other components may belong to multiple categories. In addition, TLC profiles may be used to fingerprint markers of toxicity. However, further study is required to determine the exact toxicity markers responsible for activity.

CONCLUSION

The *Euphorbia* species investigated in this study had a similar composition of phytochemicals, except for 3 *Euphorbia ammak*, *caerulescens* and *polygona*, which in addition, showed the presence of tannins. Phytochemicals present in the species are known to possess various pharmacological activities, which support the use of *Euphorbia* species to treat various health conditions. The cytotoxicity exhibited by hexane and DCM extracts of *Euphorbia ammak*, *clavarioides* and *polygona* provide preliminary scientific evidence for their use in treatment of cancer. The clear differences in the TLC chemical profiles of the toxic and non-toxic species show the effectiveness and reliability of our methods for application as a quick screen to either verify the species or determine the toxicity of the species.

The authors are not aware of similar studies that have been conducted on these plants.

SIGNIFICANCE STATEMENT

The present study confirms the different cytotoxicity levels of *Euphorbia* species and phytochemicals present in them. It is highly important for species utilized for medicinal purposes, as the presence and levels of phytochemicals influence the efficacy and safety of the outcome of treatment. The study further emphasizes the significance of chemical profiling of plants for use to distinguish species as well as for confirming the presence of phytochemicals of interest.

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