Scholars Academic Journal of Pharmacy
Abbreviated Key Title: Sch Acad J Pharm
ISSN 2347-9531 (Print) | ISSN 2320-4206 (Online)
Journal homepage: http://saspublishers.com

OPEN ACCESS
Pharmacy

Calendula officinalis - A Crucial and Promising Antifungal Agent with Cytotoxic Biological Attributes

Hrithik Dey[1*], Mahewish Tarannum[1], Pankaj Kumar[1], Vatsala Agarwal[1], Eliza Parvez[1], MD Naushad Ali[1], Sufia Shafieq[1], Vikas Sharma[1]

[1]Lloyd Institute of Management and Technology, Greater Noida, Uttar Pradesh, India-201306

DOI: 10.36347/sajp.2024.v13i01.004 | **Received:** 19.12.2023 | **Accepted:** 23.01.2024 | **Published:** 25.01.2024

***Corresponding author:** Hrithik Dey
Lloyd Institute of Management and Technology, Greater Noida, Uttar Pradesh, India-201306

Abstract **Original Research Article**

Globally recognized for its antifungal and anti-inflammatory properties, Calendula officinalis (Marigold) comprises various constituents, including carbohydrates, amino acids, terpenoids, glycosides, lipids, fatty acids, and carotenoids. In in-vitro assessments, it demonstrated effectiveness ranging from 54% to 79% against fungal infections, attributed to the significant presence of sesquiterpene, hydrocarbons, sesquiterpenols, δ-cadinene, α-cadinol, and epi-α-muurolol. Conversely, it also showcases robust cytotoxic activity against colon cancer, leukemia, and melanoma cells in human cancer lines, primarily due to the existence of two triterpene glycosides, calenduloside F 6¢-O-n-butyl ester, and calenduloside G 6¢-O-methyl ester.

Keywords: C. officinalis, antifungal, cytotoxic, screening, phytochemistry.

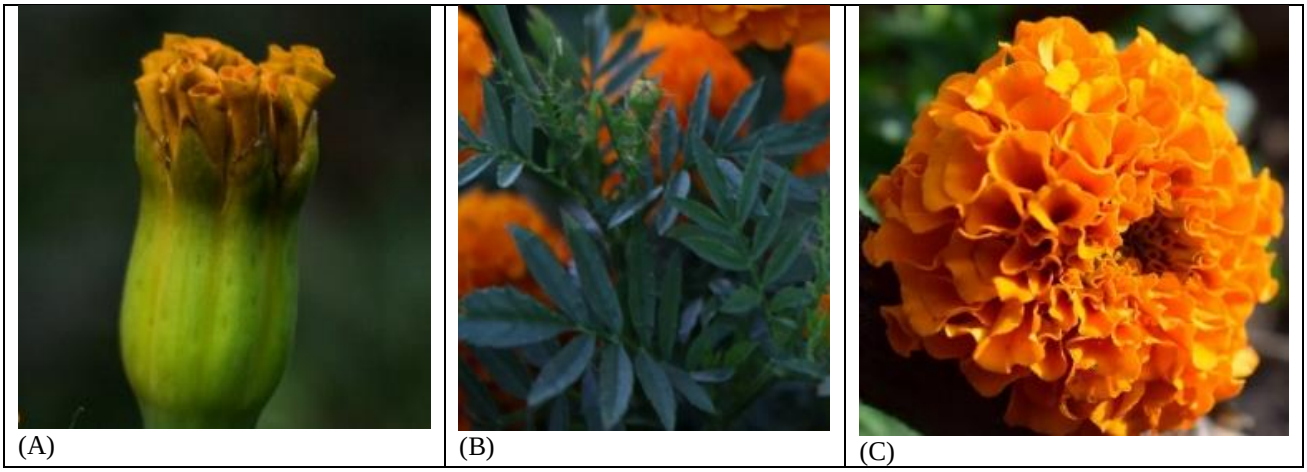
INTRODUCTION

Calendula officinalis, widely recognized as marigold, is an aromatic annual plant categorized within the Asteraceae family. It has an upright growth habit, reaching heights of up to 60 cm. The plant features angular and glandular stems, leaves measuring 2.5–7.5 cm in length, and terminal flowerheads with colors ranging from light yellow to deep orange [1]. The plant originates from Central and Southern Europe, Western Asia, and the United States, where it has been employed for medicinal purposes in countries such as China, India, the United States, and Europe [2]. As per available information, this plant variety comprises a diversity of phytochemicals, encompassing lipids, steroids, tocopherols, terpenoids, quinones, and carotenoids, offering assorted health advantages [3]. Clinical trials strongly support the healing properties of C. officinalis, which include the promotion of wound healing and the prevention of acute dermatitis [4]. Various segments of this plant have been employed for medicinal purposes across a range of conditions, with documented applications found in scientific literature and their substitutes. An active compound within the plant, known as alpha-terthienyl, serves as a nematicidal agent by diminishing plant nematodes and inhibiting the hatching of nematode eggs [5]. Furthermore, it is externally applied to address ulcers, and eczema, and facilitate wound healing. Internally, it is utilized for conditions like joint pain, irregular menstruation, abdominal pain, and dysentery. The potential medical applications of C. officinalis encompass its properties as a central nervous system stimulant, antidepressant, antioxidant, antipyretic, antidiabetic, and hypolipidemic agent [6]. With observed cytotoxic effects in in-vitro bioassay providing positive indications, it is evident that methanolic and aqueous extracts of C. officinalis (flowers) demonstrate significant bio-efficacy against myeloid cancer. The therapeutic effects are attributed to specific chemical components present in the flowers of this plant [7]. C. officinalis ethanol extract contains chemical constituents like syringic acid, quercetin, 6-hydroxykaempferol, protocatechuic acid, and quercetagetin. Notably, quercetin and 6-hydroxykaempferol, two specific compounds, demonstrated a significant reduction in the growth of HepG2 cells (Hepatocellular carcinoma) and A549 cells (lung carcinoma) [5]. Pathogens derived from both reference stocks and recently isolated strains displayed susceptibility to the antifungal properties of C. officinalis flower oil. Notably, when compared to Nystatin, a topically applied fungicide used for mucocutaneous candidiasis, this oil yielded more noteworthy results [8]. The present review article discusses the various

phytochemicals present in the plant and the antifungal and cytotoxic activity exhibited by the plant.

PHYTOCHEMISTRY

Phytochemical screening studies have identified prominent classes of chemical compounds, including terpenoids, flavonoids, coumarins, quinones, volatile oils, carotenoids, amino acids, and more [2].



(A) (B) (C)

Fig 1: A. Calendula officinalis (young flower bud) [9]; B. Calendula officinalis (leaf) [10]; C. Calendula officinalis (full flower) [11]

Volatile oil

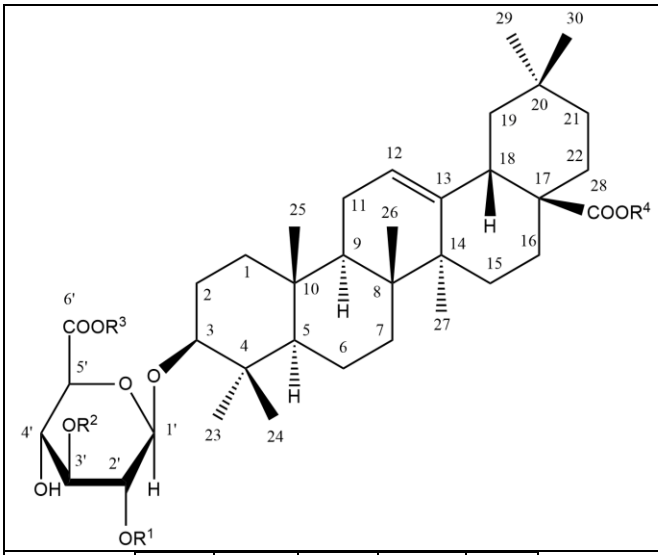
The concentration of volatile oil in C. officinalis flowers peaks at 0.97% during the full flowering stage and reaches its lowest point at 0.13% during the pre-flowering stage. Moreover, the composition of the volatile oil exhibits varying patterns across different stages of the vegetative cycle. Various monoterpenes and sesquiterpenes, such as α-thujene, sabinene, limonene, 1,8-cineol, p-cymene, trans-β-ocimene, terpene-3-carene, nonanal, terpene-4-ol, 3-cylohexene-1-ol, phellandrene, α-terpineol, and geraniol, contribute to the plant's age-dependent characteristics. During post-flowering periods, the essential oils show reduced levels of p-cymene and elevated concentrations of α-cadinene, α-cadinol, T-murolol, limonene, and 1,8-cineol. These essential oils find applications as flavoring agents in chewing gums, candy, and pharmaceuticals, as well as fragrance agents in cosmetics. Notably, T-muurolol and α-cadinol exhibit antimicrobial properties [12].

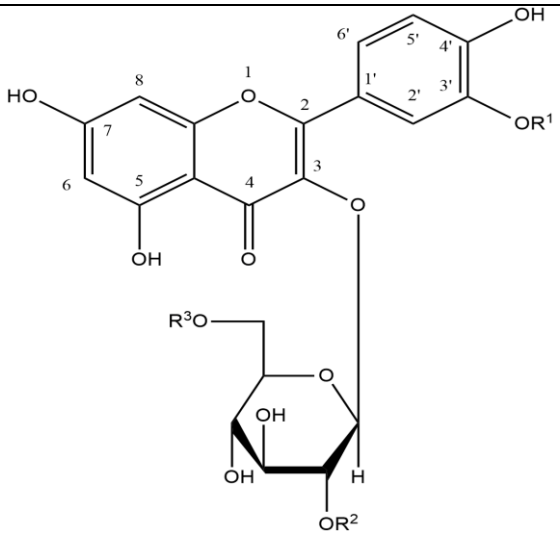
Flavonoids

Several flavonoids (flavanol glycosides) were extracted from an n-BuOH fraction of an ethanol (or methanol) extract of marigold flowers. These include isorhamnetin 3-O-neohesperidoside, isorhamnetin-3-O-2G-rhamnosylrutinoside, isorhamnetin 3-O-rutinoside, quercetin 3-O-glucoside, rutin, isoquercitrin, neohesperidoside, isorhamnetin-3-O-neohesperidoside, isorhamnetin-3-O-2G-rhamnosylrutinoside, isorhamnetin-3-O-rutinoside, quercetin-3-O-glucoside, and quercetin-3-O-rutinoside, isorhamnetin, and isoquercetin [13].

Terpenoids

The petroleum ether extract derived from C. officinalis flowers contains various terpenoids, encompassing diesters of diols [14], sitosterols, stigmasterols [15], and 3-monoesters of taraxasterol [16]. Among the identified compounds, several tri-terpene glycosides are present, including calendula glycoside A (1), calendula glycoside A 6¢-O-methyl ester (2), calendula glycoside A 6¢- O-n-butyl ester (3), calendula glycoside B (4), calendula glycoside B 6¢-O-n-butyl ester (5), calendula glycoside C (6), calendula glycoside C 6¢-O-methyl ester (7), calendula glycoside C 6¢-O-n-butyl ester (8), calenduloside F 6¢-O-n-butyl ester (9), and calenduloside G 6¢-O-methyl ester (10). Tri-terpenes 1-9 exhibit inhibitory effects on 12-Otetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice, and all show potential cancer chemopreventive activities against a 60-cell-line human cancer panel. Additionally, the discovery of carnosic acid acetate, a novel tri-terpenic ester of the oleanane series, was made in the flower extract [17].





	R^1	R^2	R^3	R^4
1	Glc	Gal	H	Glc
2	Glc	Gal	Me	Glc
3	Glc	Gal	n-Bu	Glc
4	Glc	Gal	H	H
5	Glc	Gal	n-Bu	H
6	H	Gal	H	Glc
7	H	Gal	Me	Glc
8	H	Gal	n-Bu	Glc
9	H	H	n-Bu	Glc
10	H	Gal	Me	H
11	Me	Rha	H	
12	Me	Rha	Rha	
13	Me	H	Rha	
14	H	H	H	
15	H	H	Rha	

Gal = B-D-galactopyranosyl
Glc = B-D-glucopyranosyl
Rha = a-L-rhamnopyranosyl
n-Bu = n-butyl
Me = methyl

Coumarins

The ethanol extract from the inflorescence of C. officinalis comprises the coumarins scopoletin, umbelliferone, and esculetin [18].

Quinones

In C. officinalis, various quinones were identified, including plastoquinone, phylloquinone, and α-tocopherol in the chloroplast, ubiquinone, phylloquinone, and α-tocopherol in the mitochondria, as well as phylloquinone in the leaves [19].

Amino acids

The ethanol extract from the flowers of the plant is reported to contain 15 amino acids in free form, including alanine, arginine, aspartic acid, asparagine, valine, histidine, glutamic acid, leucine, lysine, proline, serine, tyrosine, threonine, methionine, and phenylalanine. In terms of amino acid content, the leaves contain approximately 5%, stems 3.5%, and flowers 4.5% [20].

Carotenoids

Various carotenoids were identified in the methanol extract of C. officinalis flowers, specifically in the leaves, petals, and pollen. The carotenoids neoxanthin, 9Z-neoxanthin, violaxanthin, luteoxanthin, auroxanthin, 9Z-violaxanthin, flavoxanthin, mutatoxanthin, 9Z-anthroxanthin, and lutein were observed in both pollen and petals. The total carotenoid content in petals was determined to be 7.71%, while in pollen, the total carotenoids measured 1.61% [21].

PHARMACOLOGICAL ACTIVITIES

Cytotoxic Activity

Lutein is present in significant quantities in Calendula officinalis, and studies have associated its presence with a reduced likelihood of cancer, cardiovascular disease, and age-related macular degeneration (AMD) [22]. The therapeutic effects of essential oils from plants differ from those of their isolated major compounds. Research indicates that marigold essential oil demonstrates stronger anticancer effects on NB4 and EACC cell lines compared to isolated compounds [23]. Marigold essential oils exhibit cytotoxicity against diverse tumor cell lines, indicating their potential for cancer treatment without causing harm to healthy cells [24]. A549 and HEPG2 cells were inhibited by Quercetin and 6-hydroxykaempferol at a concentration of 50 g/mL, while A549 cells were

inhibited by protocatechuic acid and quercetagetin [25]. The cytoprotective activity in crude extracts was most pronounced in the flavonoid fraction abundant in quercetin and quercetagetin. However, high doses of patuletin were found to exert cytotoxic effects on Jurkart cells [26]. The in-vitro ethyl acetate soluble fraction of calendula flower extract, containing two primary compounds, calenduloside G'6-O-methyl-ester and calenduloside F'6-O-butyl-ester, displayed cytotoxic effects. Calenduloside G'6-O-methyl-ester showcased anti-cancer properties against melanoma, leukemia, and colon cancer (HCC-2998) cell lines. Additionally, calenduloside F'6-O-butyl-ester demonstrated anticancer effects on these cell lines. Moreover, aqueous laser-activated calendula extract (LACE) inhibited the proliferation of murine and human tumor cell lines by 70–100% through cell cycle arrest at the G0/G1 phase and caspase-3-induced apoptosis in vitro. Furthermore, LACE exhibited anti-cancer activity in mice [27]. Furthermore, the methanol extract derived from C. officinalis flowers has exhibited cytotoxic activity in vitro within the ethyl acetate soluble fraction [13]. Moreover, it has been found that various extracts from the plant's leaves, flowers, and the entire body show cytotoxic effects on MRC5, HeP2, and ascetic cells from Ehrlich carcinoma. The saponin-rich portion of these extracts also exhibited in-vivo antitumor activity in the Ehrlich mouse carcinoma model [28].

Antifungal Activity

Calendula officinalis demonstrates efficacy against a variety of bacteria and fungi [29]. The essential oils derived from the plant exhibited notable antifungal properties against a range of fungi, including Candida dubliniensis, Candida albicans, Candida guilliermondii, Candida parapsilosis, Candida glabrata, Candida tropicalis, and Candida krusei [8]. The methanolic extracts of the plant were observed to have susceptibility against Botrytis cinerea, Fusarium moniliforme, and Pythium ultimum as well [30]. The flower extracts, formed using different solvents, display distinct levels of activity against various microorganisms [31]. Aqueous extracts of the calendula flower demonstrated greater sensitivity against Streptococcus aureus compared to ethanolic, methanolic, and petroleum ether extracts. This suggests that aqueous extracts possess more potent antibacterial properties [32]. The organic extracts from the plant exhibited growth inhibition and antifungal activity against most Fusarium spp., although they did not show similar effects against Aspergillus spp [33].

MATERIALS AND METHODS

Fresh flowers from Calendula officinalis were gathered and carefully examined. The plant underwent a thorough cleaning with water before being sun-dried. Once dried, the plant was finely ground into a coarse powder. This powder underwent extraction through maceration using 80% aqueous ethanol over 3 days. This extraction process was repeated three times to ensure the separation of all phytochemicals from the plant. The solvent was then filtered, and the resulting filtrate was subjected to evaporation using a rotary evaporator until a dense brown extract was obtained [34]. The extract was preserved in vials at a temperature of 4°C for future utilization.

Antifungal test:

The disc diffusion method was employed for this procedure. The previously prepared extract was diluted to a concentration of 10 mg/ml using an appropriate solvent. A filter paper was saturated with 30 µL of the prepared extract and then positioned onto the inoculated potato dextrose agar (PDA) medium. A positive control was established by impregnating another filter paper with 30 µL of fluconazole. The plates were subsequently incubated for 1-2 days at 37°C. Antifungal activity was assessed by measuring the zone of inhibition (ZOI) in millimeters and comparing it with the control [35].

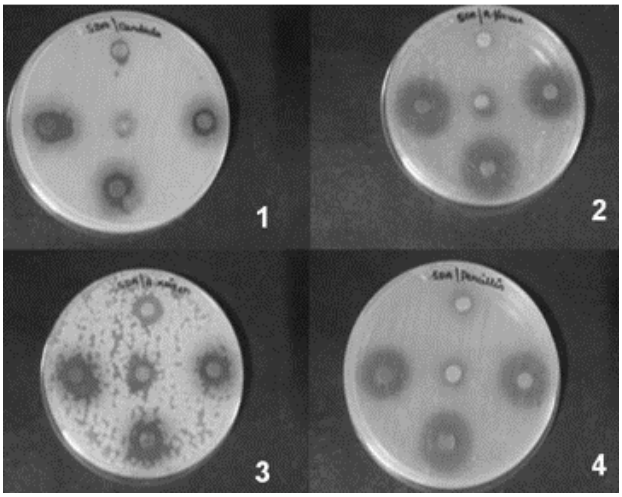


Fig 2: Zone of Inhibition in agar disc diffusion method; Fungal strains used: 1. Candida albicans, 2. Aspergillus niger, 3. Aspergillus flavus, 4. Penicillium chrysogenum [5]

Cytotoxicity test:

The MTT assay, utilized for assessing cytotoxicity by measuring cellular metabolic activity, was conducted in this study. 24-well plates were employed, each plated with approximately (1 × 105 MCF7 cells/well). These plates were then incubated at 37°C in a 5% CO2 atmosphere. Calendula officinalis extract served as the test control, and 5-Fluorouracil was used as the positive control. Both test and reference samples underwent serial dilution and were added to pre-marked wells. One well was treated solely with the diluent, serving as the negative control, while the remaining wells received the extract and 5-Fluorouracil. The plates were incubated again at 37°C for 24 hours in a 5% CO2 atmosphere. Following incubation, cells were removed from the wells, washed with PBS buffer, and subjected to an additional 4-hour incubation after the addition of specific reagents. Subsequently, 1 ml of DMSO was added to each well, and absorbance was measured at 570 nm using a UV-visible spectrophotometer. The results were then graphically represented.

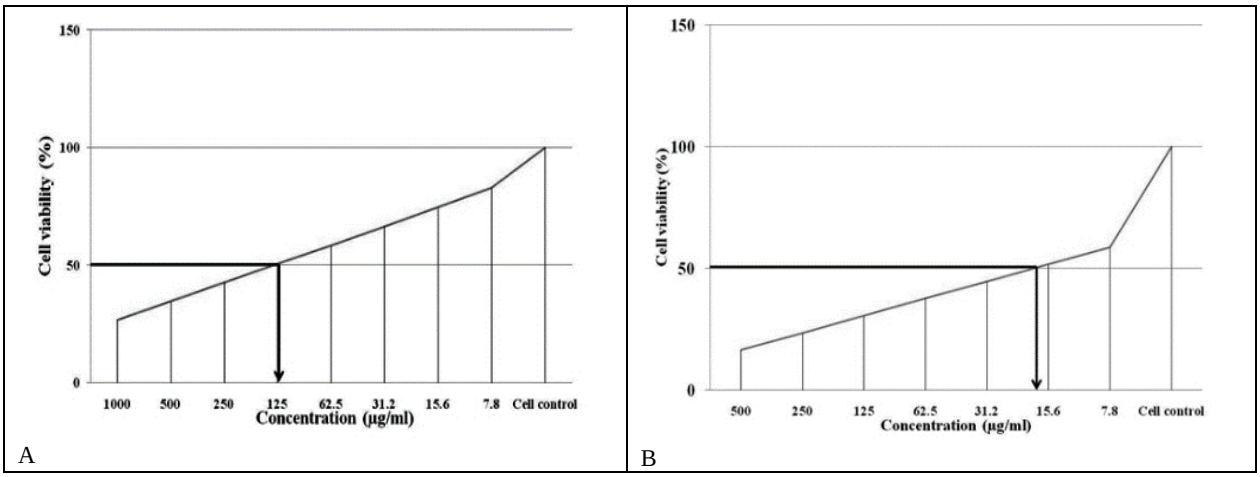


Fig 3: (A) MTT assay and cell viability in MCF-7 cells with Tagetes erecta petal extract; (B) MTT assay and cell viability in MCF-7 cells with 5-Fluorouracil [5]

RESULT

The disc diffusion method was employed to assess the antifungal activity of the C. officinalis extract against four fungal strains, as detailed in Table 1.

Table 1: Antifungal activity of C. officinalis extract and fluconazole [5]

Fungal strains	1000 µg/ml C. officinalis extract ZOI (mm)	1mg/ml Fluconazole ZOI (mm)
Penicillium chrysogenum	18	10
Candida albicans	15	7
Aspergillus niger	18	14
Aspergillus flavus	21	12

The extract exhibited effective antifungal activity even at lower concentrations when compared to fluconazole, targeting the four fungal strains.

The MTT assay was employed to observe the cytotoxic effect of the plant. The IC50 for C. officinalis extract was determined to be 125 µg/ml, while for 5-Fluorouracil, it was found to be 15.6 µg/ml, as illustrated in Table 2 [5].

Table 2: MTT assay of C. officinalis extract and the resultant cell viability [5]

Concentration (µg/ml)	Cell viability (%) C. officinalis extract	Cell viability (%) Fluconazole
1000	26.71	959
500	34.59	16.70
250	42.78	23.59
125	50.66	30.71
62.5	58.34	37.93
31.2	66.53	44.61
15.6	74.71	51.83
7.8	82.90	58.62
Control	100	100

CONCLUSION

This review encompasses an exploration of diverse sources of Calendula officinalis and the associated phytochemical constituents, including terpenoids, flavonoids, coumarins, quinones, volatile oils, carotenoids, and amino acids, among others. The antifungal and cytotoxic effects of the plant's methanol and ethanol extracts were discussed, with comparisons made to standard samples such as fluconazole and 5-fluorouracil. Notably, the extract demonstrated effective antifungal activity at low concentrations against four fungal strains compared to fluconazole. The MTT assay was employed to assess the plant's cytotoxicity, revealing an IC50 of 125 µg/ml for C. officinalis extract and 15.6 µg/ml for 5-Fluorouracil.

REFERENCES

1. Khan, M. U., Rohilla, A., Bhatt, D., Afrin, S., Rohilla, S., & Ansari, S. H. (2011). Diverse belongings of Calendula officinalis: an overview. *Int J Pharm Sci Drug Res*, *3*(3), 173-177.
2. Muley, B. P., Khadabadi, S. S., & Banarase, N. B. (2009). Phytochemical constituents and pharmacological activities of Calendula officinalis Linn (Asteraceae): a review. *Tropical journal of pharmaceutical research*, *8*(5).
3. John, R., & Jan, N. (2017). Calendula Officinalis-An Important Medicinal Plant with Potential Biological Properties. *Proc Indian Natl Sci Acad*, 93.
4. D'Ambrosio, M., Ciocarlan, A., Colombo, E., Guerriero, A., Pizza, C., Sangiovanni, E., & Dell'Agli, M. (2015). Structure and cytotoxic activity of sesquiterpene glycoside esters from Calendula officinalis L.: Studies on the conformation of viridiflorol. *Phytochemistry*, *117*, 1-9.
5. VA, V. V., Xavier, A. S., & David, D. C. (2019). In-vitro evaluation of antifungal and anticancer properties of Tagetes Erecta petal extract. *Biomedical and Pharmacology Journal*, *12*(2), 815-823.
6. Shetty, L. J., Sakr, F. M., Al-Obaidy, K., Patel, M. J., & Shareef, H. (2015). A brief review on medicinal plant Tagetes erecta Linn. *Journal of Applied Pharmaceutical Science*, *5*(3), 91-95.
7. Abudunia, A. M., Marmouzi, I., Faouzi, M. E. A., Ramli, Y., Taoufik, J., El Madani, N., ... & Ibrahimi, A. (2017). Anticandidal, antibacterial, cytotoxic and antioxidant activities of Calendula arvensis flowers. *Journal de Mycologie Médicale*, *27*(1), 90-97.
8. Gazim, Z. C., Rezende, C. M., Fraga, S. R., Svidzinski, T. I. E., & Cortez, D. A. G. (2008). Antifungal activity of the essential oil from Calendula officinalis L.(Asteraceae) growing in Brazil. *Brazilian Journal of Microbiology*, *39*, 61-63.
9. https://unsplash.com/photos/KgRPNtP7CQg. Accessed 3 March 2023
10. https://unsplash.com/photos/2j8X-RpB1sM. Accessed 3 March 2023
11. https://unsplash.com/photos/KgRPNtP7CQg. Accessed 3 March 2023
12. Okoh, O. O., Sadimenko, A. A., & Afolayan, A. J. (2007). The effects of age on the yield and composition of the essential oils of Calendula officinalis. *Journal of Applied Sciences*, *7*(23), 3806-3810.
13. Ukiya, M., Akihisa, T., Yasukawa, K., Tokuda, H., Suzuki, T., & Kimura, Y. (2006). Anti-inflammatory, anti-tumor-promoting, and cytotoxic activities of constituents of marigold (Calendula officinalis) flowers. *Journal of natural products*, *69*(12), 1692-1696.
14. Wilkomirski, B., & Kasprzyk, Z. (1979). Free and ester-bound triterpene alcohols and sterols in cellular subfractions of Calendula officinalis flowers. *Phytochemistry*, *18*(2), 253-255.
15. Adler, G., & Kasprzyk, Z. (1975). Free sterols, steryl esters, glucosides, acylated glucosides and water-soluble complexes in Calendula officinalis. *Phytochemistry*, *14*(3), 627-631.
16. Wiłkomirski, B. (1985). Pentacyclic triterpene triols from Calendula officinalis flowers. *Phytochemistry*, *24*(12), 3066-3067.
17. Naved, T., Ansari, S. H., Mukhtar, H. M., & Ali, M. (2005). New triterpenic esters of oleanene-series from the flowers of Calendula officinalis Linn. *Org Chem Incl Med Chem*, 44, 1088-1091.
18. Kerkach, A. I., Komissarenko, N. F., & Chernobai, V. T. (1986). Coumarines of the inflorescences of Calendula officinalis and Helichrysum arenarium. *Khim Prir Soed*, 6, 777-777.
19. Janiszowska, W., Michalski, W., & Kasprzyk, Z. (1976). Polyprenyl quinones and α-tocopherol in Calendula officinalis. *Phytochemistry*, *15*(1), 125-127.
20. Abajova, R. L., Aslanov, S. M., & Mamedova, M. E. (1994). Amino acids of calendula officinalis. *Chemistry of Natural Compounds*, *30*(15), 641-641.
21. Goodwin, T. W. (1954). Studies in carotenogenesis: the carotenoids of the flower petals of calendula officinalis. *Biochem J*, 58, 90-94.
22. Wang, M., Tsao, R., Zhang, S., Dong, Z., Yang, R., Gong, J., & Pei, Y. (2006). Antioxidant Activity, Mutagenicity/Anti-Mutagenicity, and Clastogenicity/Anti-Clastogenicity of Lutein from Marigold Flowers. *Food and Chemical Toxicology*, 44, 1522-1529.
23. Mahmoud, G. I. (2013) Biological Effects, Antioxidant and Anticancer Activities of Marigold and Basil Essential Oils. *Journal of Medicinal Plants Research*, 7, 561-572.
24. De-Oliveira, P. F., Alves, J. M., Damasceno, J. L., Oliveira, R. A. M., Dias, H. J., Crotti, A. E. M., & Tavares, D. C. (2015) Cytotoxicity Screening of Essential Oils in Cancer Cell Lines. *Revista Brasileirade Farmacognosia*, 25, 183-188.
25. Lu, H., Yang, S., Ma, H., Han, Z., & Zhang, Y. (2016) Bioassay-Guided Separation and Identification of Anticancer Compounds in TageteserectaL. *Flowers. Analytical Methods*, 8, 3255-3262.

26. Chkhikvishvili, I., Sanikidze, T., Gogia, N., Enukidze, M., Machavariani, M., Kipiani, N., ... & Rodov, V. (2016). Constituents of French Marigold (Tagetes patula L.) flowers protect jurkat t-cells against oxidative stress. *Oxidative medicine and cellular longevity*, *2016*.
27. Jiménez-Medina, E., Garcia-Lora, A., Paco, L., Algarra, I., Collado, A., & Garrido, F. (2006). A new extract of the plant Calendula officinalis produces a dual in vitro effect: cytotoxic anti-tumor activity and lymphocyte activation. *BMC câncer*, *6*, 1-14.
28. Boucaud-Maitre, Y., Algernon, O., & Raynaud, J. (1988). Cytotoxic and antitumoral activity of Calendula officinalis extracts. *Die Pharmazie*, *43*(3), 220-221.
29. Iauk, L., Lo Bue, A. M., Milazzo, I., Rapisarda, A., & Blandino, G. (2003). Antibacterial activity of medicinal plant extracts against periodontopathic bacteria. *Phytotherapy research*, *17*(6), 599-604.
30. Mares, D., Tosi, B., Poli, F., Andreotti, E., & Romangnoli, C. (2004). Antifungal Activity of Tagetuspatulaon Some Phytopatogheni Fungi Ultraestructural Ev idence on Phythumultimum. *Microbiological Research*, 859, 295-304.
31. Padalia, H., & Chanda, S. (2015). Antimicrobial efficacy of different solvent extracts of Tagetes erecta L. flower, alone and in combination with antibiotics. *Applied Microbiology: open access*, *1*(1), 1-10.
32. Roopashree, T. S., Dang, R., Rani, S. R. H., & Narendra, C. (2008). Antibacterial activity of antipsoriatic herbs: Cassia tora, Momordica charantia and Calendula officinalis. *International Journal of Applied research in Natural products*, *1*(3), 20-28.
33. Thembo, K. M., Vismer, H. F., Nyazema, N. Z., Gelderblom, W. C. A., & Katerere, D. R. (2010). Antifungal activity of four weedy plant extracts against selected mycotoxigenic fungi. *Journal of applied microbiology*, *109*(4), 1479-1486.
34. Bashir, S., Janbaz, K. H., Jabeen, Q., & Gilani, A. H. (2006). Studies on spasmogenic and spasmolytic activities of Calendula officinalis flowers. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, *20*(10), 906-910.
35. Efstratiou, E., Hussain, A. I., Nigam, P. S., Moore, J. E., Ayub, M. A., & Rao, J. R. (2012). Antimicrobial activity of Calendula officinalis petal extracts against fungi, as well as Gram-negative and Gram-positive clinical pathogens. *Complementary Therapies in Clinical Practice*, *18*(3), 173-176.