Anti-Microbial Study of Marigold Leaves

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***Abstract*—The alarming rise in antimicrobial resistance has intensified the search for novel, plant-based therapeutic agents with broad-spectrum efficacy. Plants from the *Tagetes* genus, commonly known as marigolds, are widely used in traditional medicine for their antimicrobial, anti- inflammatory, and antioxidant properties. While much of the focus has been placed on marigold flowers, the leaves remain relatively underexplored despite their potential to contain potent bioactive compounds. This study aims to evaluate the antimicrobial activity of ethanolic extracts from *Tagetes spp*. leaves against selected pathogenic bacterial strains, including *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, and Candida albicans*. Leaf samples were shade-dried, ground, and subjected to ethanol extraction using cold maceration. Antimicrobial efficacy was assessed via agar well diffusion assay and zone of inhibitory determination. The results demonstrated that the marigold leaf extract showed promising antibacterial activity, particularly against Gram-positive bacteria, with inhibition zones ranging from moderate to high. Phytochemical screening revealed the presence of flavonoids, alkaloids, phenolics, saponins, and tannins, which are compounds known to contribute to antimicrobial mechanisms. These findings indicate that *Tagetes spp*. leaves could serve as a cost-effective, natural source of antibacterial agents.**

***Index Terms*—broad-spectrum efficacy, antimicrobial, anti-inflammatory, anti-oxidant properties**

1. INTRODUCTION

The resurgence of herbal medicine in contemporary healthcare systems has garnered significant attention, driven by a myriad of factors including historical efficacy, cultural practices, rising healthcare costs, and an increasing demand for natural and holistic treatment options. Herbal medicine, which involves the use of plant-based substances to treat and prevent various ailments, has been a cornerstone of human health practices for millennia. This essay delves into the reasons behind the growing trend of using herbal drugs in recent times, highlighting their benefits, challenges, and the scientific advancements that support their use.

Marigold (*Tagetes and Calendula species*) has been used traditionally due to its antimicrobial activity, with several studies validating its efficacy against a variety of pathogens. Marigold's antimicrobial activity is due to its high phytochemical content, such as flavonoids, carotenoids, and other bioactive compounds (1–3).

Marigold leaf and flower extracts have shown antibacterial activity against a number of clinically significant pathogens. For example, methanolic extracts of marigold flowers and leaves partially inhibited Serratia marcescens, Enterobacter cloacae, and Alcaligenes faecalis (4).Marigold extracts also showed weak antibacterial action against skin and wound-infesting bacteria Staphylococcus aureus and Pseudomonas aeruginosa, and the yeast Candida albicans (2).

In summary, marigold leaves and flowers are rich in different bioactive compounds responsible for their antimicrobial activity. Although the antimicrobial activity of marigold extracts per se can be moderate, it can be improved when they are coupled with additional technologies, for instance, nanoparticle

synthesis or loading into nanofiber scaffolds, and can find practical applications in wound healing and pathogen management (2,4).

1.1 Overview of Antimicrobial Agents

Antimicrobial agents are an important weapon against infectious diseases and against microbial contamination in different areas. Recent focus has been placed on creating new antimicrobial materials to fight the growing antimicrobial resistance (AMR) issue.

Chitosan, being a biopolymer, has also been discovered due to its wide-spectrum antimicrobial activity, biocompatibility, and biodegradability). Its activity is physicochemical property- and microorganism-type-dependent. Carbon dots (CDs) also have extensive prospects as antimicrobial agents due to their distinctive optical properties and enhanced biocompatibility (5).

Amyloid peptides that were once linked to neurodegenerative diseases have been discovered to be antimicrobial. The peptides can disrupt microbial membranes, induce pathogens to agglutinate, and potentially be used to develop new antimicrobial drugs (6). Metal nanoparticles such as silver-coordinated N-heterocyclic carbene complexes have also been reported to demonstrate tremendous potential as antimicrobial agents that can release active Ag^+ cations for a sustained period of time (7,8).

Overall, the area of antimicrobial research is underway, with a focus on innovation in the next generation of antibiotics as well as research on new materials to counter AMR. This development promises smarter better means of tackling the global issue of antimicrobial resistance. Various studies have investigated active antimicrobial compounds against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli*, and *Candida albicans*. Various approaches, from bacteria, nanoparticles, to plant extracts, have been found to inhibit the pathogens.

A novel strain of Bacillus subtilis showed antibacterial activity against S. aureus and E. coli (9). Likewise, 2-phenyl-quinoline-4-carboxylic acid derivatives were also reported to show good antibacterial activity against *S. aureus* and *E. coli*, among which compound showed the maximum inhibition (10). Surprisingly, tetracyclic 1,4-benzothiazine derivatives showed more activity than the standard drug fluconazole against *C. albicans* (11).

Nanoparticles are a promising tool against such microorganisms. Gold nanoparticles were observed to lyse Gram-negative and Gram-positive bacteria (12). Silver nanoparticles exhibited maximum antimicrobial activity (80-100%) against all four pathogens compared to gold nanoparticles (13). TiO_2 nanoparticles prepared by the sol-gel method exhibited activity against *S. aureus, E. coli*, and *C. albicans* under visible light (14). Pseudomonas aeruginosa biosynthesized zinc oxide nanoparticles exhibited excellent activity against all four pathogens with different minimum inhibitory concentrations (15).

1.2 Objective of this Study

The study aimed to assess the antimicrobial activity of marigold (*Tagetes*) leaf extracts (22). The study had the following aims in particular:

Evaluate the phenolic profile, β-carotene, and flavonoid contents variation of *Tagetes erecta* and *Tagetes patula* leaf extracts. Research studied the antioxidant activity using DPPH free radical scavenging and linoleic acid peroxidation inhibition assays. It also evaluated the antibacterial and antifungal activities by disc diffusion and resazurin microtiter-plate assays against different bacterial and fungal strains (22).

Not. Ably, the sample analysis revealed that both T. erecta and T. patula extracts exhibited promising antimicrobial activity against Bacillus subtilis and Alternaria alternata when compared with positive controls (ciprofloxacin and flumequine). Furthermore, the methanol extract of T. patula contained the highest total phenolic (35.8 mg GAE/g) and total flavonoid (16.9 mg CE/g) content. T. erecta extract had the maximum DPPH radical-scavenging activity (IC50 5.73 μg/mL) and linoleic acid peroxidation (80.1%) inhibition (22).

The research proved that marigold leaf extracts are good sources of polyphenols with superior biological activities, such as having major antimicrobial activity. This work indicates the prospects of marigold leaves as natural antimicrobial substances, which may be useful for medicinal purposes and agriculture (22).

2. BIOCHEMICAL PROFILE OF MARIGOLD LEAVES

2.1 Introduction

Tagetes or marigolds, which are indigenous to the Americas, have become globally cultivated for their aesthetics, medicinal properties, and agricultural purposes (3). Well-known species such as *T. minuta, T. erecta, T. patula, and T. tenuifolia* find particular importance because of their versatility of use (3). Their flowering heads are full of carotenoids like lutein esters, for which they are highly sought in food, animal nutrition, and pharmaceuticals (23).

Aside from their good looks, marigolds also have a rich history in traditional medicine, where they've been applied for dental issues, digestive issues, and even emotional and neurological disorders (3). Studies also point out their antioxidant and anti-inflammatory potential, as well as their capacity to block some enzymes (3). Here, farmers love marigolds for their organic pest- and disease-control capabilities, which mean they are a viable option for protecting crops in a sustainable manner (3).

Now, researchers are discovering even more about these incredible flowers researching where they derive their bright colours (21), employing intelligent technology to enhance their farming (24), and even experimenting with reusing mushroom waste as fertilizer to assist them in growing (25). These advances demonstrate the enormous potential marigolds have across sectors, from agriculture to medicine.

2.2 Plant Description

Marigold, known scientifically as *Calendula officinalis L. and Tagetes erecta L.*, represents one of nature's most versatile gifts to humanity. As a proud member of the Asteraceae family (10,16), this vibrant plant has captivated cultures worldwide under various names - from the familiar pot marigold to the striking African marigold (figure 2).

Figure 1: Marigold leaves.

The plant's cheerful blossoms, ranging from sunny yellow to deep orange, owe their radiant hues to nature's masterful chemistry. These colours emerge from complex carotenoid pigments (21), creating not just visual beauty but also signalling the plant's rich biochemical potential.

For centuries, marigold has occupied a special place in traditional medicine cabinets across civilizations. Modern science now confirms what ancient healers long understood - the plant's remarkable anti-inflammatory and antibacterial properties (2,26). These validated therapeutic effects bridge the gap between folk wisdom and evidence-based medicine.

Beyond its medicinal and ornamental value, marigold reveals surprising environmental superpowers. Research demonstrates its capacity for phytoremediation, effectively absorbing and containing heavy metals like lead and cadmium (27). Farmers increasingly value marigold as a natural protector, with studies showing its effectiveness against soil-borne diseases and its potential to safeguard tobacco crops from bacterial wilt through strategic intercropping (21).

2.3 Chemical Constitutes of Plant

Marigold leaves contain a diverse array of bioactive compounds (table 1) that contribute to their medicinal, pesticidal, and nutritional properties. While less studied than the flowers, the leaves exhibit a unique phytochemical profile with significant biological activities.

Table 1:List of Chemical Constituents obtain from different parts of the plan and their use.

Category	Examples	Plant Part	Biological Activity / Use
Carotenoids	, Zeaxanthin, β-Carotene, Lycopene	Flowers	Antioxidant, vision protection, food colouring
Flavonoids	uercetin, Kaempferol, Isoquercetin, Rutin	Flowers, leaves	Antioxidant, anti- inflammatory, anti-cancer
ssential Oils	one, Ocimene, Linalool, Caryophyllene	Flowers, leaves	Antibacterial, antifungal, insect repellent
Terpenoids	enyl, Thymol, Carvacrol, Terpinolene	Leaves, flowers	Antifungal, insecticidal, fragrance
Phenolic Acids	c acid, Ferulic acid, Gallic acid, Vanillic acid	Whole plant	Antioxidant, antimicrobial
Tannins	drolysable and condensed tannins	Leaves, stems	Astringent, antimicrobial
Saponins	Saponin glycosides (non-specific)	Roots, stems	Antimicrobial, immune- modulating
Alkaloids	Trace alkaloids (less studied in *Tagetes*)	Whole plant (low)	ntially anti-inflammatory or analgesic

Table 2: Marigold Vernacular Names

Language / Region	Vernacular Name	Species Usually Referred
English	Marigold	*Tagetes erecta*, *T. patula*
Hindi	Genda	*Tagetes erecta*
Bengali	Gada Phool	*Tagetes erecta*
Malayalam	Chamanthi	*Tagetes erecta*
Gujarati	Galgota	*Tagetes erecta*
Marathi	Jhendoo Phool	*Tagetes erecta*
Punjabi	Genda	*Tagetes erecta*
Nepali	Sayapatri	*Tagetes erecta*

2.4 Botanical Description

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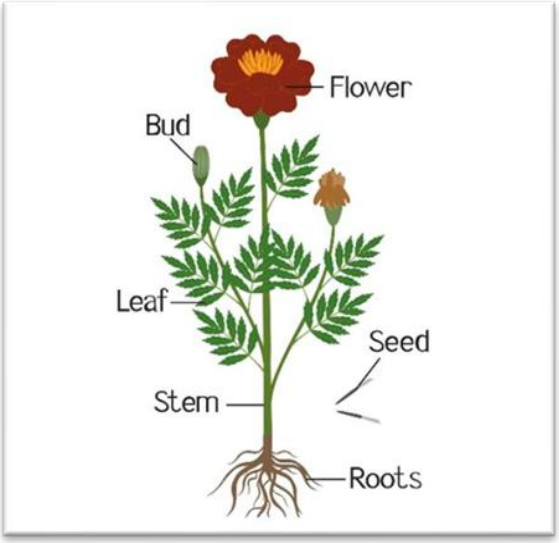

Figure 2: Parts of Marigold Tree.

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(24) and even experimenting with reusing mushroom waste as fertilizer to assist them in growing (25). These advances demonstrate the enormous potential marigolds have across sectors, from agriculture to medicine.

2.5 Marigold: Bridging Traditional Wisdom and Modern Applications

For centuries, marigold (*Tagetes spp*.) has been revered across cultures not only for its vibrant beauty but also for its remarkable therapeutic properties. This multifaceted plant continues to captivate researchers as traditional uses find validation through modern scientific investigation.

2.5.1 Healing Traditions Meet Modern Medicine

Rooted in ancient practices, marigold has served as a cornerstone in traditional healing systems worldwide (26). In Chinese medicine, practitioners have long valued its ability to clear heat, detoxify the body, support liver function, and even enhance skin and eye health (33).

2.5.2 From Folk Remedy to Clinical Application

The plant's traditional use in skin care, particularly for its anti-inflammatory and antibacterial properties (2) has inspired contemporary wound care innovations. Researchers are now developing advanced nanofiber scaffolds incorporating marigold extracts for improved wound dressing materials.

2.5.3 Beyond Medicine: Agricultural and Nutritional Value

Marigold's benefits extend far beyond human health. Farmers have harnessed its natural pesticidal properties, with studies confirming its effectiveness against fungi, bacteria, and insects (3). Particularly noteworthy is α-terthienyl from marigold roots, which shows promise in controlling plant-parasitic nematodes (34).

3. MATERIALS AND METHODS

3.1 Plant Material Collection

Fresh and healthy leaves of marigold (*Tagetes spp*.) were collected from the Gorola region of Birbhum district, West Bengal, India, during the early morning hours to preserve their phytochemical integrity. The plant was identified based on its morphological characteristics such as leaf shape, flower colour, and arrangement. Only mature and undamaged leaves were selected to ensure consistency and quality of the extract. The collected plant material was placed in clean, dry paper bags and transported to the laboratory under ambient conditions for further processing. This region is known for its cultivation of ornamental and medicinal plants, and the marigold species collected are commonly used in traditional therapeutic practices, which further supports their selection for the present study.

3.2 Drying of Plant Material

After collection, the marigold (*Tagetes spp*.) leaves were thoroughly washed with clean tap water to remove dust, soil particles, and other surface contaminants. The cleaned leaves were then spread evenly on clean blotting sheets and subjected to shade drying for a period of five days. The drying process was carried out in a well-ventilated area, away from direct sunlight, to preserve the heat-sensitive phytoconstituents and prevent the degradation of active compounds. The leaves were regularly turned over to ensure uniform drying and to avoid fungal growth. Upon complete drying, the leaves became crisp and brittle, indicating the removal of moisture and readiness for the next stage of extraction.

3.3 Grinding plant leaves

Once the marigold (*Tagetes spp*.) leaves were completely dried, they were subjected to size reduction to facilitate efficient extraction of phytochemicals. The dried leaves were first broken into smaller fragments by hand and then ground into a fine powder using a

Figure 3: Powder of leaves.

mechanical grinder. The grinding process was carried out under clean and dry conditions to prevent contamination. The resulting leaf powder was sieved through a fine mesh to obtain uniform particle size and then stored in airtight, labelled containers at room temperature until further use. This powdered form of the plant material ensured maximum surface area exposure during the subsequent extraction process.

3.4 Extraction

Extraction methods have changed tremendously over time, with emphasis placed on creating greener and more effective processes. Liquid extraction methods, including ambient ionization mass spectrometry, allow direct analysis of intricately complex samples with no specific pretreatment, accelerating analysis while maintaining molecular localization (36). Green extraction methods (GreETs) have been developed as eco- friendly alternatives to traditional methods, seeking to enhance selectivity and sensitivity and minimize adverse effects on the operator and environment (37).

Interestingly, new methods such as three-liquid-phase solvent extraction enable the separation of three metal ions in a single operation, while standard two-liquid-phase systems need at least two operations (38). Furthermore, dispersive solid phase extraction (dSPE) is also of interest owing to its simplicity, reduced extraction time, and minimum solvent use, while being highly efficient and applicable across a broad range (33).

In summary, recent developments in the extraction methods have centred on enhancing efficiency, minimizing environmental effects, and increasing analytical power. Extraction methods like microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and rapid solid-liquid dynamic extraction (RSLDE) have proven to have benefits over conventional methods, such as shorter extraction times, enhanced yields, and less energy usage (37). These innovations are especially useful in a range of fields such as biological, food, and environmental research, providing a more sustainable and efficient method for sample preparation and analysis**.**

3.5 Soxhlet extraction

Soxhlet extraction was employed to obtain the ethanolic extract of marigold (*Tagetes spp*.) leaves. Initially, 50 grams of dried and powdered marigold leaves were accurately weighed and packed into a cellulose thimble. The thimble was then placed inside the main chamber of the Soxhlet extractor. A solvent system comprising 500 mL of 70% (v/v) ethanol prepared by mixing ethanol and distilled water in a 70:30 ratio—was used as the extracting solvent. The extraction process was conducted over a continuous period of 24 hours under gentle reflux conditions.

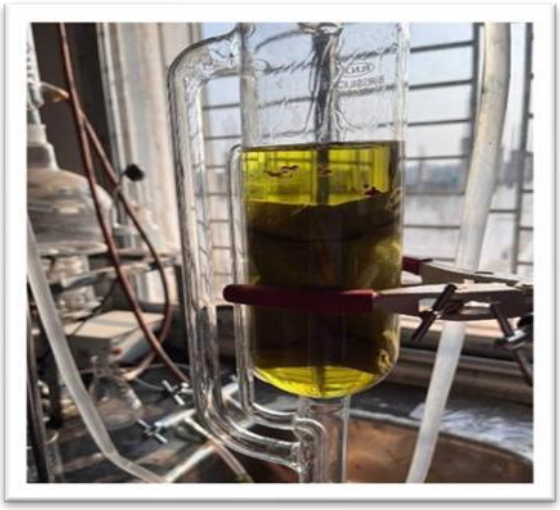

Figure 4: Soxhlation Process.

During this time, approximately 10 to 12 siphon cycles occurred, ensuring thorough percolation and solvent recirculation through the plant material. To optimize extraction efficiency, the solvent was pre-heated and maintained at a temperature close to its boiling point (approximately 78°C), allowing the ethanol vapours to condense and drip onto the plant material repeatedly. The cycle enabled the continuous dissolution of phytoconstituents into the solvent. Upon completion of the extraction, the dark green-coloured crude extract was carefully collected and allowed to cool at room temperature before proceeding to the next steps of filtration and concentration.

3.6 Filtration of Crude Extract

Following the completion of the Soxhlet extraction process, the crude ethanolic extract obtained was subjected to filtration in order to remove any residual

plant debris and undissolved particulates. The extract was first allowed to cool to room temperature to prevent damage to the filter paper and to ensure safe handling. Filtration was carried out using Whatman No. 1 filter paper, which was carefully placed in a clean glass funnel and positioned over a sterile conical flask or round-bottom flask. The filtration process was conducted under gravity, without applying any external pressure or vacuum, to maintain the integrity of the extract and avoid any contamination. The crude extract was poured slowly into the funnel, allowing the solvent to pass through the filter paper at a steady rate. This step effectively separated the solid residues from the liquid portion of the extract. The filtrate collected in the round-bottom flask was then allowed to stand undisturbed for approximately 30 minutes. This resting period enabled the sedimentation of any fine suspended particles that may have passed through the filter, thereby enhancing the clarity and quality of the final extract. The clear supernatant was then carefully decanted or transferred for further concentration and drying.

3.7 Drying of Extract

Following the completion of Soxhlet extraction, the resulting filtrate, which contained a concentrated mixture of phytochemicals dissolved in 70% ethanol, was subjected to a drying process to remove the solvent and obtain a stable crude extract. Initially, the extract was filtered through Whatman No. 1 filter paper to eliminate any remaining particulates and plant debris. The clear filtrate was then transferred to clean glass trays and placed in a hot-air oven maintained at a controlled temperature of 45 ± 2°C. This gentle drying temperature was selected to prevent degradation of thermolabile compounds and to allow slow evaporation of the solvent. The drying process continued until the liquid fraction was sufficiently evaporated, resulting in the formation of a thick, viscous, semi-solid green mass. The obtained crude extract represented a yield of approximately 7.5% w/w based on the initial weight of the dried leaf powder. Once dried, the extract was carefully scraped from the trays using a sterile spatula, weighed, and transferred into sterile amber-coloured Eppendorf tubes with a 2 mL capacity. The use of amber tubes was crucial to protect the bioactive constituents from light-induced degradation. The
samples were then sealed and stored at 4°C in a refrigerator until required for further phytochemical screening and biological assays.

Figure 5: Drying of Extraction.

4. PHYTOCHEMICAL SCREENING

Phytochemical analysis of plant extracts such as marigold leaves usually yields a variety of bioactive compounds. Although the context given here does not directly refer to marigold leaves, we can infer from the context of similar research on other plants on how to proceed and what to expect in phytochemical analysis.

Phytochemical screening usually detects the occurrence of different secondary metabolites like alkaloids, flavonoids, phenolics, tannins, terpenoids, steroids, saponins, and glycosides ((39– 41). They are usually detected using qualitative assays, thin-layer chromatography (TLC), and more sophisticated methods like Gas Chromatography-Mass Spectrophotometry (GC-MS) (40,42).

Interestingly, various plant parts can have varying levels of these compounds. In certain species, for example, the bark is reported to have the highest total phenolic content (TPC), whereas the leaves contain the highest total flavonoid content (TFC) (40).This emphasizes the need to check different plant parts independently during phytochemical screening.

The availability of these phytochemicals is usually coupled with other bioactivities. For instance, flavonoids and phenols are antioxidant in nature (43). It has been demonstrated by research that ethyl acetate fractions of the leaves of some plants possess considerable antioxidant activity with high

correlation between total phenolic content and antioxidant capacity (43).

In summary, although actual figures regarding marigold leaves are not given, phytochemical screening would most probably identify a variety of bioactive compounds comparable to those present in other plant crops. These can include alkaloids, flavonoids, phenolics, tannins, and terpenoids, among others. Such compounds may or may not be present and the amount may be influenced by the method of extraction and the plant part used for the analysis. Additional research employing methods such as FTIR and GC-MS would offer more precise information regarding the individual compounds found in marigold leaves.

4.1 Test for Alkaloids

Minute quantity of extract free from solvent i.e. chloroform, alcohol and water were taken and stirred primarily with a little drop of dil. HCL followed by filtration. The filtrate collected was
subjected to treatment with an array of alkaloid reagents. Chemical tests performed for the presence of alkaloids were given below.

Table 3: Chemical test for the presence of Alkaloid.

Sl. No.	Test Name	Method	Result
1	Mayer's Test	1 mL of extract was mixed with 1 mL of Mayer's reagent.	Cream or reddish-brown precipitate indicating the presence of alkaloids.
2	endorff's Test	1 mL of extract was mixed with 1 mL of Dragendorff's reagent.	red precipitate confirming the presence of alkaloids.
3	Wagner's Test	1 mL of extract was mixed with 1 mL of Wagner's reagent.	Appearance of reddish-brown coloured precipitate indicating the presence of alkaloids.

4.2 Test for Glycosides

Small amount of extract was dissolved in distilled water separately followed by filtration. Then the filtrate is treated with dil. HCl and were hydrolysed for 2 to 4 hours on water bath different preliminary tests were performed for the presence of glycosides. Chemical tests performed for the presence of cardiac glycoside are given below.

Table 4: Chemical test for the presence of Glycosides.

Sl. No.	Test Name	Method	Result
1.	Bontrager's Test	Treat 0.5 mL extract with 1 mL 1 N HCl, heat, then shake with equal volume chloroform	ink/red colour in aqueous layer the presence of Anthocyanins glycosides.
2.	Keller-Killiani Test	Treat 0.5 mL extract with 1 mL glacial acetic acid + drop $FeCl_3$, underlayer with conc. H_2SO_4	Brown ring at interface

4.3 Test for Tannins

Table 5: Chemical test for the presence of Tannins.

Sl. No.	Test Name	Method	Result
1.	Ferric Chloride Test	nL extract with few drops 1% $FeCl_3$	ue-black or brownish- green color

4.4 Test for Phenols

Table 6: Chemical test for the presence of Phenols.

Sl. No.	Test Name	Method	Result
1.	Ferric Chloride Test	Mix 0.5 mL extract with 2 drops 5% $FeCl_3$	Blue-black or greenish colour

4.5 Test for Saponins

Table 7: Chemical test for the presence of Saponins.

Sl. No.	Test Name	Method	Result
1.	Foam Test	Dilute 1 mL extract with 5 mL distilled water, shake vigorously for 2 min	Stable foam (~1 cm height)

4.6 Test for Terpenoids

Table 8: Chemical test for the presence of Terpenoids.

Sl. No.	Test Name	Method	Result
1.	Salkowski Test	Mix 0.5 mL extract with 2 mL chloroform + 2 mL conc. H_2SO_4	Reddish-brown interface

Test for Flavonoids

Table 9: Chemical test for the presence of Flavonoids.

Sl. No.	Test Name	Method	Result
1.	Shinoda Test	Add a few Mg turnings and 1 drop conc. HCl to 0.5 mL extract	Pink/red colour

4.7 Test for Steroids

Table 10: Chemical test for the presence of Steroids.

Sl. No.	Test Name	Method	Result
1.	Liberman- Burchard Test	Mix 0.5 mL extract with 1 mL chloroform + 1 mL acetic anhydride, add a few drops conc. H_2SO_4	Blue-green ring

COMPARATIVE ANTIMICROBIAL EVALUATION OF MARIGOLD LEAF EXTRACT AND CHLORAMPHENICOL USING THE AGAR DISC DIFFUSION METHOD

4.8 Microorganism species used

The bacterial strains (ATCC, CDL, Kolkata) used were *E. coli* (ATCC 8739), *B. Subtilis* (ATCC 6633), *Candida albicans* (ATCC 10231), *Staphylococcus aureus* (ATCC 6538). The bacterial strains discussed in the question overlap with a number of the microorganisms utilized in the studies detailed in the given papers. Below is a recap of the pertinent information:

E. coli ATCC 8739, *B. subtilis* ATCC 6633, *C. albicans* ATCC 10231, and *S. aureus* ATCC 6538 were all employed in different antimicrobial assays throughout the studies. *E. coli* ATCC 8739 and *S. aureus* ATCC 6538P were specifically tested against physalis isolated from Physalis angulata L. (44). *C. albicans* ATCC 10231 was utilized in various studies, such as assays against physalis (44), silver nanoparticles (45), zinc oxide nanoparticles (46), *Nigella sativa* essential oil (47), and *Juniperus thurifera L*. essential oils (48).

Notably, although *B. subtilis* ATCC 6633 was not specifically indicated in the given contexts,

B. subtilis DSM 6333 was employed in antimicrobial assays with *Nigella sativa* and *Juniperus thurifera* essential oils (47). This indicates that various strains of *B. subtilis* are typically utilized in antimicrobial research.

Briefly, the mentioned bacterial strains in the question are being commonly employed for antimicrobial work, especially for natural product and nanoparticle testing. Both Gram-positive (*S. aureus, B. subtilis*) and Gram-negative (*E. coli*) bacteria and fungi (*C. albicans*) are included, thus a wide range is available for assessment of antimicrobial activity.

4.9 Preparation of Nutrient Agar Media

4.9.1 Nutrient Agar

Nutrient agar is a general-purpose medium used for the cultivation of non-fastidious bacteria. It contains peptone, beef extract, sodium chloride, and agar, providing essential nutrients for microbial growth. Common in laboratories, it supports colony formation and is ideal for routine bacterial culture and microbiological studies.

Preparation nutrient agar

To prepare nutrient agar, dissolve 5 g peptone, 3 g beef extract, 5 g sodium chloride, and 15 g agar in 1 litter of distilled water. Heat the mixture while stirring until all components are fully dissolved. Adjust the pH to around 7.4 using dilute acid or base if necessary. Sterilize the solution by autoclaving at 121°C for 15 minutes. After sterilization, cool the medium to about 45–50°C and pour into sterile Petri dishes for solidification and subsequent microbial inoculation.

Table 11: Composition of Nutrient Agar.

Typical Formula	Nutrient Agar (g/L)
Beef Extract	5 g
Distilled Water	1000 ml
Peptone	5 g
Sodium Chloride	3 g
Agar	25 g

4.9.2 Preparation

a) Weigh the ingredients and mix them in 1 litter of distilled water.
b) Heat the mixture gently while stirring to dissolve the components completely.
c) Adjust pH to 7.4 if necessary, using NaOH or HCl.
d) Sterilize by autoclaving at 121°C for 15 minutes.
e) Cool to 45–50°C before pouring into sterile Petri dishes.

4.10 Inoculation of bacterial suspensions

i. Sterile Petri plates are taken and numbered as 1, 2, 3, 4, 5, and 6.
ii. Nutrient agar medium, cooled to about 45°C after autoclaving, is poured into each sterile Petri plate to form a thick 6 mm layer. The plates are then left undisturbed for 2 hours to allow solidification.
iii. After solidification, under aseptic conditions (in a laminar airflow chamber), 0.5 ml of bacterial suspension from test tube 1 is taken and inoculated into Petri plate 1.
iv. Similarly, 0.5 ml of bacterial suspensions from test tubes 2 to 6 are inoculated into Petri plates 2 to 6, respectively.
v. Once inoculated, wells are made in the agar using sterile cork borers.
vi. The prepared plates are placed in a BOD

incubator and incubated at 37°C for 72 hours for bacterial growth observation.

4.11 Preparation of Test and Standard Solutions

The crude extract of marigold leaves was dissolved in distilled water and diluted with sterile distilled water to obtain concentrations of 20, 30, and 40 mg/mL. Similarly, standard amoxicillin was prepared at the same concentrations (20, 30 and 40 mg/mL) using sterile distilled water. All solutions were freshly prepared before each experiment.

4.12 Agar Disc Diffusion Method

The agar disc diffusion test is a popular method for antimicrobial susceptibility testing (AST) of many types of microorganism. The test is done by placing antibiotic-impregnated discs on agar cultures inoculated with the test organism and measuring the inhibition zones produced after incubation.

A number of studies have contrasted the disc diffusion technique with other AST methods and identified both the advantages and the disadvantages. In the case of *Elizabethkingia anophelis*, the disc diffusion technique had considerable differences from the reference agar dilution method, with highly significant error rates of more than 1.5% for nine antibiotics (49). Also, in the case of Malassezia species, the disc diffusion technique was not able to accurately forecast minimum inhibitory concentration (MIC) values, and thus may not be a good alternative method for antifungal susceptibility testing of these yeasts (50).

But the disc diffusion method was found to have good performance in some organisms and antibiotics. In the case of Neisseria gonorrhoeae, it had a high categorical agreement (>90%) with agar dilution and Etest methods for extended-spectrum cephalosporins (51). In the case of cefiderocol testing of Acinetobacter baumannii, disc diffusion on iron-depleted Mueller-Hintonagar plates had higher categorical agreement (95.1%) with the reference broth microdilution method than commercial Mueller-Hinton agar plates (52). Finally, the reliability of the agar disc diffusion method depends on the organism and antibiotic to be tested. Although it is a valuable screen for some purposes, caution should be exercised in viewing limitations, and interpretations must be made carefully in the case of some challenging-to-test organisms or emergent antibiotics. In some cases, alternative techniques or standard protocol modifications are required to ensure greater accuracy.

4.13 Determination of Zone of Inhibition

The zone of inhibition test is a common method of measuring antimicrobial susceptibility in different microorganisms. In this method, the diameter of the clear region around an antibiotic disk applied to an agar plate seeded with the test organism is measured (53,54).A number of studies have utilized this method to assess antimicrobial activity. For example, scientists utilized the agar diffusion assay to screen the antifungal activity of clove extract-loaded microbial cellulose-based coatings against pathogens, and they noted inhibitory zones of different extract concentrations (53). In the same vein, the EUCAST disk diffusion method has been used to examine susceptibility of oral anaerobes to antibiotics, with inhibition zones measured after certain incubation times (55).

Notably, however, the zone of inhibition technique can also pose difficulties in some instances. For instance, when cefiderocol susceptibility was being tested, extensive areas of technical uncertainty were observed with contemporary EUCAST breakpoints (56). Moreover, routine solvents such as DMSO and DMF, which are frequently employed to dissolve new antimicrobial compounds, have themselves been shown to have inhibitory activities against microbial growth and can lead to false-positive findings if not suitably managed (57). In summary, although the zone of inhibition technique is popular and useful in antimicrobial susceptibility testing, researchers should appreciate possible limitations and variability across various organisms and antimicrobial agents. Satisfactory controls, established protocols, and attention to organism-specific growth needs are crucial for proper interpretation of results (55,57,58).

5. DISCUSSION

5.1 Qualitative Phytochemical Screening of Merigold leaf (alcoholic) extract

The qualitative phytochemical screening of the alcoholic extract of *Tagetes* (marigold) leaves revealed the presence of several secondary metabolites. Positive results were observed for alkaloids (Dragendorff's and Wagner's tests), glycosides (Borntrager's and Killer-Killiani tests),

phenols and tannins (Ferric Chloride test), saponins (Foam test), and terpenoids (Salkowski test), indicating the potential medicinal value of the extract. However, negative results were recorded for steroids (Liebermann-Burchard test), and flavonoids (Shinoda test), suggesting their absence or presence in insignificant amounts.

Table 12: Result of Qualitative Phytochemical Screening.

Phytochemical Test	Specific Test Used	Result
Alkaloids	Mayer's Test	Negative
	Dragendorff's Test	Positive
	Wagner's Test	Positive
Glycosides	Borntrager's Test	Positive
	Killer-Killiani Test	Positive
Phenols and Tannins	Ferric Chloride Test	Positive
Flavonoids	Shinoda Test	Negative
Saponins	Foam Test	Positive
Steroids	Liebermann-Burchard Test	Negative
Terpenoids	Salkowski Test	Negative

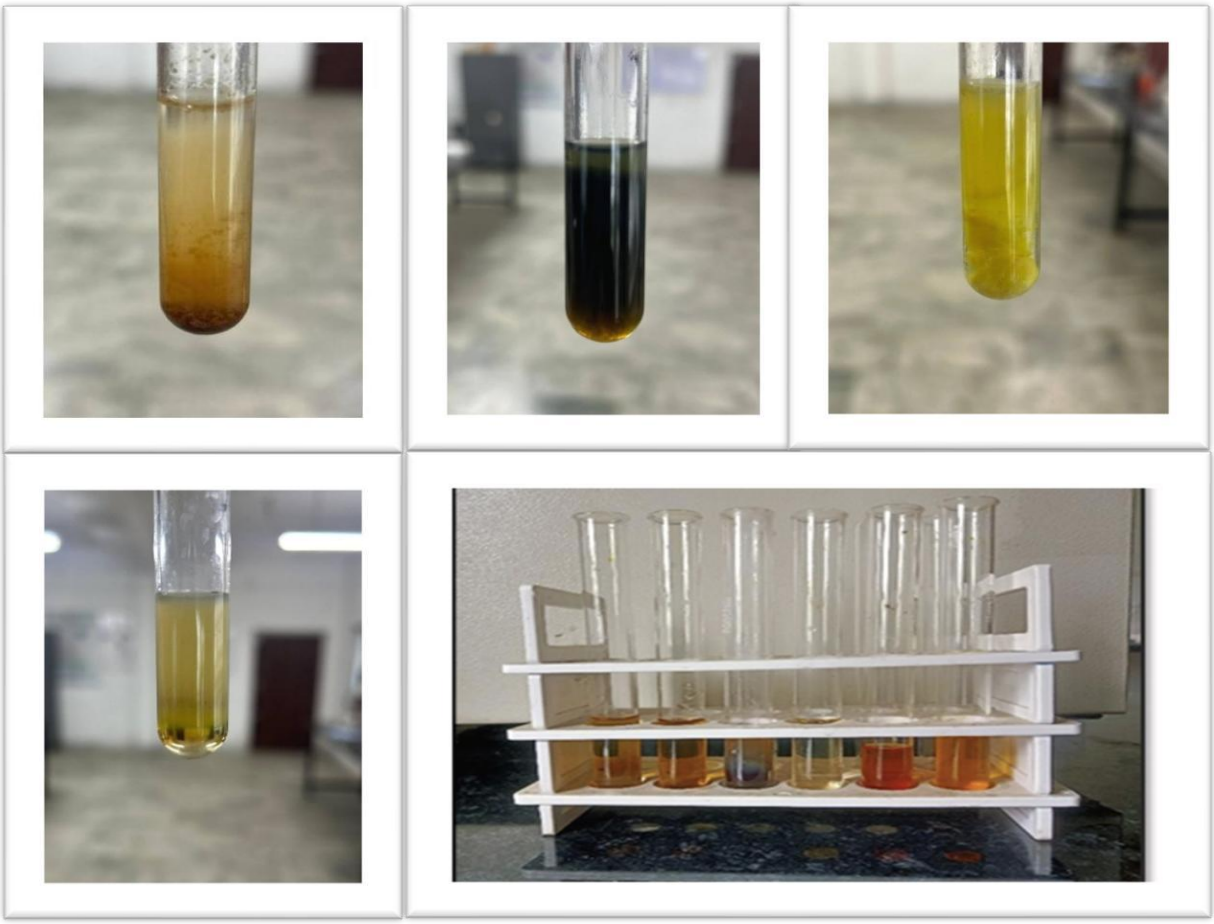

Figure 8: Test of Qualitative Phytochemical Screening.

5.2 Evaluation of Antimicrobial Activity of Chloramphenicol Standard Solution

a) The zone of inhibition produced by Chloramphenicol standard solution is found to be 40mm with concentration 30mg/ml for *Bacillus subtilis*.

b) The zone of inhibition produced by Chloramphenicol standard solution is found to be 20mm with concentration 20mg/ml for *Candida albicans*.

c) The zone of inhibition produced by Chloramphenicol standard solution is found to be 35mm with concentration 40mg/ml for *Staphylococcus aureus*.

d) The zone of inhibition produced by Chloramphenicol standard solution is found to be 42mm with concentration 20mg/ml for *E. coli.*

5.3 Evaluation of Antimicrobial activity of Alcoholic extract of Marigold leaves

a) The zone of inhibition produced by Marigold leaves solution is found to be 43mm with concentration 30mg/ml for *Bacillus subtilis*.

The zone of inhibition produced by Marigold leaves standard solution is found to be 42.5mm with concentration 30mg/ml for *Candida albicans*.

b) The zone of inhibition produced by Marigold leaves standard solution is found to be 44.2mm with concentration 30mg/ml for *Staphylococcus aureus*.

c) The zone of inhibition produced by Marigold leaves standard solution is found to be 36mm with concentration 20mg/ml for *E. coli.*

Table No 13: Result of Antimicrobial Effective Test.

Microorganism Species	oncentration (mg/mL)	Zone of Inhibition (mm)	
		Marigold leaves Extract	*Chloramphenicol*
Bacillus subtilis	20	26	22
	30	43	40
	40	29.5	28
Candida abicans	20	40.3	20
	30	42.5	18
	40	32.2	16
taphylococcus aureus	20	42	30
	30	44.2	21
	40	39	35
E. coli	20	36	42
	30	34	23
	40	30	2.2

	1	2	3	4	5	6	7	8	9	10	11	12
Microorganism Species	0			0			0			0		
Concentration (mg/mL)	20	30	40	20	30	40	20	30	40	20	30	40
Zone of Inhibition (mm) Marigold leaves Extract	26	43	29.5	40.3	42.5	32.2	42	44.2	39	36	34	30
Zone of Inhibition (mm) Chloramphenicol	22	40	28	20	18	16	30	21	35	42	23	2.2

Graph 1: Antimicrobial Effective Concentration on isolated Standard microorganism with Marigold leaves and Chloramphenicol.

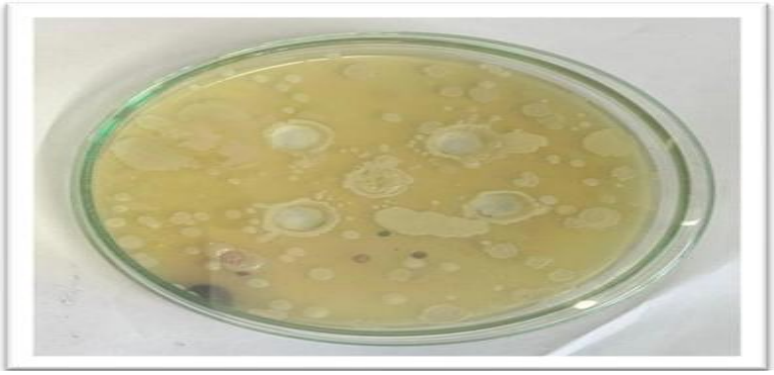

Figure 9: Growth of *E. coli, B. subtilis, C. albicans* and *S. aureus* in absence of antimicrobial agent.

Figure 11: Zone of Inhibition produced by Chloramphenicol (Standard) at concentrations of 20, 30, and 40 mg/mL against A: Bacillus subtilis, B: Candida albicans, C: Staphylococcus aureus and D: Escherichia coli.

Figure 10: Zone of Inhibition produced by Marigold leaves extraction (Standard) at concentrations of 20, 30, and 40 mg/mL against A: Bacillus subtilis, B: Candida albicans, C: Staphylococcus aureus and D: Escherichia coli.

6. RESULTS

The alcoholic extract of marigold (*Tagetes* spp.) leaves demonstrated significant antimicrobial activity against both Gram-positive and Gram-negative bacteria as well as fungal strains. In the agar disc diffusion assay, the extract showed dose-dependent inhibition zones, with the most potent effects observed at 30 mg/mL concentration. Notably, the extract exhibited stronger activity than chloramphenicol against *Bacillus subtilis* (43.0 mm vs. 40 mm) and *Staphylococcus aureus* (44.2 mm vs. 21 mm) at this concentration. Moderate activity was recorded against *Candida albicans* (42.5 mm) and *Escherichia coli* (36.0 mm). Phytochemical screening revealed the presence of alkaloids, glycosides, phenols, tannins, and saponins, which likely contribute to the antimicrobial properties. The results validate marigold leaves as a promising natural antimicrobial agent, particularly against Gram-positive pathogens, supporting its traditional medicinal use and potential applications in combating microbial infections.

7. CONCLUSION

The present study evaluated the antimicrobial potential of marigold (*Tagetes spp.*) leaf extract against a panel of pathogenic microorganisms, including *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Candida albicans*, using the agar disc diffusion assay. The extract demonstrated notable antimicrobial activity across a range of tested concentrations (20–40 mg/mL), with measurable zones of inhibition that, in some cases, were comparable to those produced by the standard antibiotic chloramphenicol. Phytochemical screening revealed the presence of several bioactive constituents such as flavonoids, tannins, phenolic compounds, saponins, and terpenoids which are well recognized for their antimicrobial properties.

These findings lend strong support to the traditional use of marigold in ethnomedicine for treating infectious conditions and position the plant as a promising natural source of antimicrobial agents. The broad-spectrum efficacy observed suggests a synergistic interaction among the phytoconstituents. Moving forward, detailed phytochemical investigations, including the isolation, structural elucidation, and bioactivity profiling of individual compounds, are warranted. Such efforts are critical in the context of the global rise in antibiotic resistance and the urgent need for novel, plant-derived therapeutic alternatives.

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