Polyphenol-Rich Purified Bioactive Fraction Isolated from *Terminalia catappa* L.: UHPLC-MS/MS-Based Metabolite Identification and Evaluation of Their Antimicrobial Potential

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Abstract: Background: Antimicrobial resistance is a major threat to humankind and the advancement of resistance due to genetic modifications and other defense mechanisms that make the current antibiotics ineffective or less efficacious. Objective: This investigation aims to isolate bioactive compounds from the leaf acetone extract of *Terminalia catappa* and to evaluate their antimicrobial potential against human pathogenic organisms. Materials and Methods: The bioactive extract was subjected to column chromatography. The fractions were assessed for their minimum inhibitory concentration, minimum fungicidal concentration, and time kill assays. UHPLC-MS/MS analysis was used to identify the bioactive molecules in the fraction. Results: The isolated fraction exhibited antimicrobial activity, with the most sensitive being *Staphylococcus aureus* (clinical isolate) and Methicillin Resistant *Staphylococcus aureus* 1503 (0.097 mg/mL), and the fungi *Trichophyton rubrum* and *Candida albicans* were inhibited at 0.097 mg/mL. The time kill assay exhibited bactericidal properties towards *S. aureus* (clinical isolate) and *Salmonella typhi* (MTCC 733). Additionally, MRSA 1503 and *Proteus vulgaris* exhibited bacteriostatic activities. The UHPLC-MS/MS analysis revealed that the fraction was rich in polyphenols. Alkaloids and some ellagitannins were identified for the first time. Conclusion: The results highlight the significant inhibition of multidrug-resistant MRSA strains and fungi by the polyphenol-rich fraction. The investigation reveals the potential use of the identified compounds for antimicrobial use, which could lower the implication of multidrug resistance.

Keywords: *Terminalia*; Combretaceae; flavonoid glycosides; time kill assay; alkaloids; MRSA; multidrug resistance; bioactive molecules; *Trichosporon asahii*; UHPLC-MS/MS

1. Introduction

The multidrug resistance (MDR) problem in the microbial world is a major challenge in treating infectious diseases [1]. Fifty percent of the world’s hospital-acquired infections are caused by MDR organisms [2]. Over two million people are infected according to World Health Organization (WHO) reports, with 2300 people dying each year. The antibiotic drug resistance problem is envisaged to outclass the cancer threat in the near future [3]. One of the most medically important pathogens is *Staphylococcus aureus*, which causes extensive community-acquired infections that are often fatal and associated with beta-lactam resistance used in hospitals. These strains are known as Methicillin-resistant *Staphylococcus aureus* (MRSA) [4]. MRSA is resistant to all beta-lactams and usually resistant to anyngoglycosides, quinolones, sulfonamides, and rifampicin, but it is usually susceptible to glycopeptides and it acquires resistance via the production of penicillin-binding protein, which lowers its affinity for beta-lactams [5]. Other resistance-acquired pathogens include *Listeria monocytogenes*, *Clostridium difficile*, *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Campylobacter jejuni*, *Neisseria gonorrhoeae*, *Vibrio cholerae*, *Salmonella* species,
Vancomycin-resistant Enterococci (VRE), and multidrug-resistant Mycobacterium tuberculosis [6]. In addition, according to the data from the center for disease control, it is estimated that, each year in United States, nearly 2 million people acquire hospital infections while 90,000 deaths occur due to it [7,8]. The majority of nosocomial infections are caused by ESKAPE pathogens, and the WHO has already listed these pathogens requiring new antibiotics [9]. Treating infections caused by the resistant pathogens becomes difficult and requires the use of more toxic and expensive drugs. The antimicrobial resistance has also been aggravated by irrational use of antibiotics in promoting the growth of livestock [10].

Fungal drug resistance is also increasing at an alarming rate, which has become a consequential menace worldwide [11]. The infections caused by dermatophytes affect the keratinized tissues that are difficult to combat, which affects both immuno-compromised and immuno-competent individuals, and treating dermatophytosis in immuno-compromised individuals is a major problem globally [12,13]. Acquired resistance to antifungal drugs includes resistance to drugs such as polyene macrolides (amphotericin B); 1,3-β-glucan synthase inhibitors (echinocandins); andazole derivatives (ketoconazole, fluconazole, voriconazole, and itraconazole), which exist in Candida species, Aspergillus species, Cryptococcus neoformans, Trichosporon beigelii, and Scopulariopsis species [14]. Trichophyton rubrum is the most widespread dermatophyte worldwide and causes 70% of dermatophytosis [15]. The advancement of drug resistance has made existing antibiotics less effective or even ineffective. To overcome the drug resistance problem, many approaches have been proposed, among which a combination of other molecules with antibiotics evidently reinstitutes the antimicrobial efficacy or may enhance the antimicrobial activity [16]. One of the possible strategies is the identification of suitable natural compounds from medicinal plants, which can be achieved by bioactivity-guided fractionation of natural compounds [17]. Natural plant products are affluent sources of antimicrobial drugs, which constitute two thirds of antibiotics used clinically [18]. Surveys conducted by the WHO report that more than 80% of the world’s population still relies on traditional medicine to treat infectious disease [19], and the fact that only 50 years of antibiotics use has led to the development of resistance reveals that more efficacious systems are needed to successfully treat infectious diseases [20]. Natural products have been used in medicine for more than 5000 years and have been explored for new drugs, and plants have played an extensive role in providing drugs, food, etc. [21]. The preliminary advantage of natural product-derived, particularly plant-derived, antimicrobials is that they do not have side effects, which is prevalent in the use of synthetic drugs [22], and they repress the growth of microorganisms via different mechanisms [23]. The major groups of phytochemical antimicrobial agents including phenolics and polyphenols, terpenoids, essential oils, and alkaloids are known to possess antimicrobial properties [24]. The biological properties of these molecules from plants have increased the interest in the field of new drugs, herbal insecticides, and antibiotics [25]. Antimicrobials from medicinal plants are a prime source of pharmaceuticals and for treating infectious diseases. Further exploration of antimicrobials from plants needs to be conducted, and weakening the side effects that are linked with synthetic antimicrobials is the need of the hour [26].

Thus, the present investigation was undertaken to isolate antimicrobial compounds from the traditional medicinal plant Terminalia catappa to provide a new understanding in combating strategies for treating infectious diseases and in developing new drugs in drug discovery research.

The Terminalia species of the Combretaceae family is a well-known ethnomedicinal plant, and the genus is reported to have more than 250 species [27]. The genus is widely distributed in Southern Asia, The Himalayas, Madagascar, Australia, and tropical and subtropical regions of Africa [28]. Different studies on the pharmacological effects of Terminalia species have reported that they have hepatoprotective activities and positive effects on immune regulation and that they improve cardiovascular diseases, diabetes, and wound healing [28]. T. catappa possess different ethnomedicinal properties like purgative,
astringent, laxative, diuretic, and leaves are used in treating dermatitis, hepatitis, and leprosy. The bark and roots are used as an antipyretic and for hemostatic purposes in Indonesia, Malaysia, and India [29,30]. The plant *T. catappa* have high levels of phytochemicals, consisting of flavonoid and their glycosides, pentacyclic triterpenoids, gallotannins, and phenolic acids [27], which may be attributed to the various biological activities, such as antifungal and antibacterial [31], antidiabetic [32], anticancer, anti-inflammatory [33], antiviral and hepatoprotective [34], aphrodisiac [35], and antioxidant activities [36]. Various species of the genus are used globally in Chinese medicine, Indian ayurvedic medicine, and traditional Tibetan medicine [37]. Considering the wide array of bioactivities and ethnomedicinal uses of *T. catappa*, the current investigation focused on the isolation of bioactive compounds and the assessment of the antimicrobial potential of the bioactive extract of the plant against human pathogenic bacteria in special reference to MRSA and bacterial clinical isolates and to dermatophytic fungi.

2. Materials and Methods

2.1. Extraction and Isolation

Healthy leaf materials were collected, washed, shade dried, and powdered in the laboratory. The powdered material was extracted with a Soxhlet apparatus using polar and non-polar solvents. The extracts were concentrated to dryness and stored until further use. Our previous study investigated the bioactivities of various extracts of *T. catappa*. During the course of our study, the acetone extract was subjected to disc diffusion assays, which showed considerably good inhibition of the test microbes. Hence, the acetone extract was subjected to column chromatography for isolation of phytocompounds.

The acetone extract (500 mg) was subjected to silica gel column chromatography (200–400 mesh) with stepwise gradient elution with ethyl acetate and methanol as the eluent system (10:0–0:10) to obtain 254 fractions of 12 mL each with a flow rate of 1 mL/min. The fractions were monitored by thin layer chromatography (TLC) to elute the phytocompounds from the fraction with ethyl acetate–methanol–water (6.2:0.9:0.6) as the eluent system and pooled up depending on the elution profile. The fractions were evaluated for their antimicrobial potential against the test pathogens. The fraction TCAF2 showed significant antimicrobial activity and was subjected to UHPLC-MS/MS for identification of the antimicrobial compounds.

2.2. Microbial Cultures and Growth Media

All of the test bacteria and fungi used in the study were procured form Microbial Type Culture Collection Center, Chandigarh, India. Six bacterial strains were used in the study: *Staphylococcus aureus* (MTCC 7443), *Salmonella typhi* (MTCC 733), clinical isolates of *Staphylococcus aureus* and *Proteus vulgaris*, and two strains of Methicillin-resistant *Staphylococcus aureus* (MRSA 1007 and MRSA 1503). The clinical isolates were provided by the Microbiology department, Mysore Medical college and Research Institute. The bacterial cultures were maintained on a Mueller–Hinton agar and in Mueller–Hinton broth (Hi-media) and stored at 4 °C until further use. The dermatophytes and yeast-like fungi, namely *Microsporum canis* (MTCC 2820), *Microsporum gypseum* (MTCC 2830), *Trichophyton rubrum* (MTCC 296), and *Candida albicans* (MTCC 183), were procured and maintained on Sabouraud-dextrose agar and Sabouraud-dextrose broth (Hi-media), and *Trichosporon asahii* (MTCC 6179) was maintained on yeast malt extract agar (Hi-media) and maintained at 4 °C. When required the test pathogens were regrown on the suitable media, incubated at optimal temperature, and used in the investigation.

2.3. Antimicrobial Assays

The MIC was established by employing a 96-well microtiter plate according to the CLSI protocol [38,39]. TCAF2 was subjected to the minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC), and the minimum fungicidal concentration (MFC) by diluting it to two-fold to obtain concentration of 5 mg/mL to 0.97 mg/mL.
The experimental setup included reference drug controls and the solvent as positive and negative controls, respectively. An aliquot of standardized inoculum was added to all the wells and incubated at respective temperatures for bacteria and fungi. The inhibition of bacterial growth was confirmed by the addition of 20 µL of an aqueous solution of 2,3,5-Triphenyltetrazolium chloride (TTC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and re-incubated for 4–5 h. The colorless well was designated as the MIC, which inhibited the growth of test microbes. A change in color indicated the presence of viable cells. MBC and MFC were ascertained by sub-culturing 10 µL of the test dilution from the lowest concentration well inoculated previously on the sterilized and solidified agar after incubation at the optimal temperatures for bacteria and fungi. The well, which gave no viable colony on the agar medium, was designated as MBC and MFC.

2.4. Time Kill Assay

The protocol of the time kill assay was adopted following [40] with slight modifications. The active fraction isolated from the acetone extract of T. catappa was set at a concentration of MIC, four-fold MIC, and eight-fold MIC. Each tube was then inoculated with a standardized suspension of clinical isolates of S. aureus and P. vulgaris, MRSA 1503, and S. typhi (MTCC 733). At prefixed time points (2, 4, 6, 8, and 24 h), the aliquots were withdrawn from each concentration and diluted serially, and the aliquots were plated on agar plates. The respective controls were included. Colony counts were performed after overnight incubation at 37 °C in ambient air. The colony counts were averaged for respective dilutions and expressed as log10 cfu/mL.

2.5. HRLCMS-Based Metabolite Profiling of TCAF2

The TCAF2 was subjected to HRLCMS analysis using an Agilent (6550 ifunnel Q-TOF’s) system consisting of a hip sampler, a binary pump, a column component, and Q-TOF with an electron ionization spray. Chromatographic separation was performed on a 1290 infinity UHPLC system fitted with a Hypersil gold column (C18X 2.1 mm-3Micron). The solution consisted of 0.1% formic acid in water (A), or 90% acetonitrile, 10% water, and 0.1% formic acid (B) as a mobile phase. The flow rate was adjusted to 0.3 mL/min with a 5 µL injection volume. The solvent system used was as follows: 0–1 min of 95% (A) and 5% (B), 1–20 min of 100% solvent (B), 20–25 min of 100% solvent (B), 25–26 min of 95% (A) and 5% (B), and 26–30 min of 95% (A) and 5% (B).

For mass detection Q-TOF, a mass spectrometer (Agilent technologies, CA, USA) was operated with dual AJS ESI (Agilent technologies, CA, USA) as an ion source and a scan range of 150–1000 M/Z. The capillary tension was set at 3500 V, the gas flow was set at 13 L/min with a 250 °C temperature. The sheath gas flow rate was 11 L/min at 300 °C. The nebulizer gas was set at 35 psi gas flow pressure. Q-TOF data acquisition and evaluation of mass spectrometry were carried out using Agilent Metlin database.

2.6. Total Flavonoid Content (TFC) Determination by Aluminium Chloride Method

The TFC was conducted following [41] with slight modifications. Five hundred microliters of TCAF2 were withdrawn from the stock solution, and 30% aqueous methanol was added. To this solution, 0.5 M aqueous sodium nitrate was added followed by the addition of a 0.5 M aluminum chloride solution. The reaction mixture was left to incubate for 5 min, and 1 M sodium hydroxide was added. The reaction mixture was mixed thoroughly, and absorbance was recorded at 510 nm. A blank was set up without the addition of the fraction. A quercetin standard curve was obtained by attaining concentrations from 1 mg/mL to 0.31 mg/mL employing the same procedure. The TFC was calculated using the regression equation, $y = 0.1065x + 1.1876$ ($R^2 = 0.8969$), where y is the absorbance of the plant extract and x is the amount of quercetin. The results were expressed as mg of QE/g of the plant extract. Triplicates were maintained, and the values are presented as...
mean ± SEM. Microsoft excel 2009 was used for statistical analysis and for generating the calibration curve.

3. Results

3.1. Inhibitory Effect of Purified Fraction on Human Pathogenic Bacteria and Fungi Assessed by Minimum Inhibitory and Minimum Bactericidal Concentration

The TCAF2 exhibited noteworthy inhibitory activity against all of the test organisms with concentrations ranging from 0.097 mg/mL to 0.390 mg/mL, with the most sensitive being *S. aureus* (clinical isolate), MRSA1503, and *S. typhi* (MTCC 733), with lowest inhibitory concentration of 0.097 mg/mL. *S. aureus* (MTCC 7443), *P. vulgaris* (clinical isolate), and MRSA1007 exhibited moderate inhibitory activity, with MICs of 0.195 mg/mL, 0.781 mg/mL, and 0.390 mg/mL, respectively, depicted in Tables 1 and 2. The MBC of the tested bacteria ranged from 6.25 mg/mL to 25 mg/mL. *S. aureus* and *P. vulgaris* (clinical isolates) exhibited MBCs at 0.39 mg/mL and 25 mg/mL, respectively. MRSA1503 and MRSA1007 exhibited bactericidal effects at 25 mg/mL and 6.25 mg/mL, respectively. *S. aureus* (MTCC 7443) and *S. typhi* (MTCC733) exhibited bactericidal property at 12.25 mg/mL and 0.78 mg/mL.

Table 1. The MIC and MBC profiles exhibited by test bacteria against TCAF2.

| Test Bacteria          | MIC (mg/mL) | MBC (mg/mL) | Gentamycin (mg/mL) |
|------------------------|-------------|-------------|---------------------|
| *S. aureus* (MTCC 7443)| 0.195       | 12.25       | 0.0019              |
| *S. typhi* (MTCC 733)  | 0.097       | 0.78        | 0.0019              |

Key: Concentration of gentamycin: 1 mg/mL.

Table 2. The MIC and MBC profiles exhibited by TCAF2 against clinical isolates and MRSA.

| Clinical Isolates | MIC (mg/mL) | MBC (mg/mL) | Ciprofloxacin (mg/mL) |
|-------------------|-------------|-------------|-----------------------|
| *S. aureus*       | 0.097       | 0.390       | 0.0019                |
| *P. vulgaris*     | 0.781       | 25          | 0.0019                |
| MRSA 1503         | 0.097       | 25          | 0.0019                |
| MRSA1007          | 0.390       | 6.25        | 0.0019                |

Key: Concentration of ciprofloxacin: 1 mg/mL.

In contrast, for the dermatophytic fungi tested, shown in Table 3, *M. canis* (MTCC 2820) and *T. rubrum* (MTCC 296) exhibited MICs at 0.097 mg/mL and *M. gypseum* (MTCC 2830) exhibited inhibitory concentrations at 0.195 mg/mL. The fractions were examined against *C. albicans* (MTCC183) and *T. asahii* (MTCC 6179), which exhibited inhibitory concentrations at 0.097 mg/mL and 1.562 mg/mL, respectively. Our results exhibited significant antifungal activity against all of the tested fungi. The minimum fungicidal concentration (MFC) of TCAF2 also exhibited very low concentrations ranging from 0.097 to 1.562 mg/mL. *M. gypseum* and *M. canis* exhibited fungicidal concentrations at 0.390 mg/mL. *T. rubrum* and *C. albicans* exhibited fungicidal concentrations at 0.097 mg/mL. *T. asahii* showed fungicidal concentrations at 1.562 mg/mL. Strong MIC and MFC were observed against all of the tested fungi.
Table 3. Effect of TCAF2 evaluated against dermatophytes and yeast-like fungi assessed by minimum inhibitory concentration and fungicidal concentration.

| Dermatophytes          | MIC (mg/mL) | MFC (mg/mL) | Amphotericin (mg/mL) | Miconazole (mg/mL) |
|------------------------|-------------|-------------|----------------------|--------------------|
| M. gypseum (MTCC 2830) | 0.195       | 0.390       | ND                   | 0.0019             |
| M. canis (MTCC 2820)   | 0.097       | 0.390       | ND                   | 0.0019             |
| T. rubrum (MTCC 296)   | 0.097       | 0.097       | ND                   | 0.0019             |
| C. albicans (MTCC 183) | 0.097       | 0.097       | 0.0019               | ND                 |
| T. asahii (MTCC 6179)  | 1.56        | 1.56        | 0.015                | ND                 |

Key: Concentration of amphotericin and miconazole: 1 mg/mL; ND: Not done.

3.2. Time Kill Assay

A time kill assay was performed against S. aureus (Figure 1a), P. vulgaris (clinical isolates) (Figure 1b), S. typhi (MTCC 733) (Figure 1c), and MRSA 1503 (Figure 1d). Though the MIC of TCAF2 gives the efficacy of the test extract, the kinetics of the antimicrobial action is limited in MIC. The time kill assay provides insight on whether the extract is bacteriostatic or bactericidal at a given concentration and at a particular time point in relation to the growth kinetics of the test bacteria.

The interaction between the bacteria and TCAF2 was assessed at MIC, four-fold MIC, and eight-fold MIC. The tested concentrations exhibited various kinetics against the test bacteria at different time points. The most susceptible was S. typhi (MTCC 733), which exhibited rapid bactericidal activity after 2 h of exposure at eight-fold MIC with 3.39 log10 cfu/mL relative to the drug-free control. The results were consistent with the MBC determined. At four-fold MIC, the killing of S. typhi (MTCC 733) was observed after the eighth hour of contact with 3.09 log10 cfu/mL, but regrowth was observed after 24 h with 2.59 log10 cfu/mL viable colonies. At MIC, the maximum reduction in viable colonies was observed to be 2.25 log10 cfu/mL at 6th hour of contact time. The kill kinetics for S. typhi increased with concentration, and rapid killing was observed at a higher MIC. S. aureus (clinical isolate) when exposed to TCAF2 bactericidal activity was achieved after 4 h of drug interaction at eight-fold MIC, and no viable bacterial colonies were observed, indicating a bactericidal effect. The bacterial cells continued to reduce at all of the concentrations tested (MIC, four-fold MIC, and eight-fold MIC). However, when the cells reached the 24th hour, the cell density increased at MIC to 2.57 log10 cfu/mL, which is in accordance with the MIC assay revealing the bacteriostatic activity. At four-fold MIC and eight-fold MIC, the cells almost vanished after 24 h, indicating a bactericidal effect of TCAF2.

The TCAF2 exerted a bacteriostatic effect at all of the tested concentrations against Proteus vulgaris (clinical isolate) with a maximum log reduction of 1.67 log10 cfu/mL. Maximum reduction was achieved at all of the tested concentrations after 8h of contact time, but regrowth was observed until the 24th hour of incubation. Bacteriostatic activity progressed until the 24th hour of interaction at MIC and four-fold MIC, which exhibited log reductions of 0.75 and 0.85 log10 cfu/mL, respectively, when compared with the drug-free control having a viable cell count of 6.17 log10 cfu/mL.

MRSA 1503 exhibited bacteriostatic properties against all of the tested concentrations. The rate at which MRSA 1503 was inhibited increased with an increase in concentration. Maximum log reduction was achieved at eight-fold MIC with 2.4 log10 cfu/mL at 2 h of contact time. However, the viable cells continued to increase after the 4th hour until the 24th hour, but the viable cells were drastically reduced compared with the drug-free control, with 6.32 log10 cfu/mL, which confirms the bacteriostatic property at eight-fold MIC. At MIC and four-fold MIC, maximum reduction was achieved at 2 h and 4 h of contact time, with 1.98 log10 cfu/mL and 1.96 log10 cfu/mL, respectively, and the cells continued to increase until the 24th hour, suggesting bacteriostatic activity at all of the tested concentrations. Rapid and maximum inhibition of the MRSA 1503 was observed...
after 4 h of contact time at all of the tested concentrations, indicating that the extract exhibited time-dependent reduction for the test bacteria.

**Table 3.** Effect of TCAF2 evaluated against dermatophytes and yeast-like fungi assessed by minimum inhibitory concentration and fungicidal concentration.

| Dermatophytes | MIC (mg/mL) | MFC (mg/mL) | Amphotericin (mg/mL) | Miconazole (mg/mL) |
|---------------|-------------|-------------|----------------------|--------------------|
| M. gypseum (MTCC 2830) | 0.195 | 0.390 | ND | 0.0019 |
| M. canis (MTCC 2820) | 0.097 | 0.390 | ND | 0.0019 |
| T. rubrum (MTCC 296) | 0.097 | 0.097 | ND | 0.0019 |
| C. albicans (MTCC 183) | 0.097 | 0.097 | 0.0019 | ND |
| T. asahii (MTCC 6179) | 1.56 | 1.56 | 0.015 | ND |

Key: Concentration of amphotericin and miconazole: 1 mg/mL; ND: Not done.

**Figure 1.** Time–kill curves of TCAF2 treated with different concentrations against (a) *S. aureus* (clinical isolate), (b) *P. vulgaris* (clinical isolate), (c) *S. typhi* (MTCC 733), and (d) MRSA 1503.

**3.3. UHPLC-MS/MS Analysis and Total Flavonoid Content Estimation**

The phytochemical profile of TCAF2 was analyzed by UHPLC-ESI-MS/MS analysis. The LCMS chromatogram of the positive (Figure 2) and negative ionization modes (Figure 3), and the high-resolution mass spectroscopy directed the detailed evaluation and identification of 32 important phytocompounds. The most abundant class of phytochemicals identified was the flavonoids and its glycosides (13), ellagitannins (1), gallotannins (4), phenolic acids (7), alkaloids (4), and proanthocynidin (1), lignan glucoside (1), triterpenoid (1), and galloyl glucose (1) and are presented in Tables 4 and 5. The alkaloids, and some ellagitannins and condensed tannin were not reported in *Terminalia catappa*. The total flavonoid content estimated in TCAF2 was found to be 129 ± 0.001 mg QE/g of the plant extract. As depicted in Tables 4 and 5, the fraction was dominated by flavonoids compared with any other phytochemical identified in the fraction.
Figure 2. Positive ion chromatogram of TCAF2.

Figure 3. Negative ion chromatogram of TCAF2.
### Table 4. Ultrahigh-performance liquid chromatography and MS/MS analysis in negative mode of TCAF2 isolated from the leaf acetone extract of *Terminalia catappa*.

| Sl. No | Rt (Min) | Identified Compound | Class of Compound | Formula | [M – H]− | MS/MS Fragment Ions | Mass | Reference |
|-------|----------|---------------------|-------------------|---------|----------|---------------------|------|-----------|
| 1     | 1.108    | 1-O-Galloyl fructose Gallotannin | C13H16O10 | 331.06 | 211.02, 271.045, 169.012, 125.22 | 332.03 | [42] |
| 2     | 3.267    | Punicacortin D | Gallotannin | C48H28O30 | 541.02 | 300.996 | 1084.06 | [43] |
| 3     | 3.935    | 2,6-Digalloyl glucose | Gallotannin | C20H20O14 | 483.076 | 169, 271.044 | 484.03 | [43] |
| 4     | 3.985    | Castacrenin D | Ellagitannin | C48H30O30 | 541.02 | 781, 601.99, 300.99 | 1804.06 | [43] |
| 5     | 4.432    | Punicacortin B | Gallotannin | C27H22O18 | 541.024 | 300.91 | 634.07 | [43] |
| 6     | 4.606 | 2-O-Ferruloylhydroxyxictric acid Phenolic acid | C16H16O11 | 383.06 | 384.06 | - | [44] |
| 7     | 4.78 | 1,3,6-Trigalloyl glucose | Galloyl glucose | C27H24O18 | 635.087 | 483.0, 635.08, 300.993, 161.01 | 636.09 | [45] |
| 8     | 5.069    | Kaempferol 3-O-β-D-galactoside | Flavone glycoside | C21H20O11 | 447.092 | 447.089, 285.03 | 448.1 | [46] |
| 9     | 5.249    | Gallic acid 3-O-(6-galloylglucose) | Galloylglucose | C20H20O14 | 483.069 | 129, 169, 313 | 484.07 | [47,48] |
| 10    | 5.483    | Ellagic acid | Phenolic acid | C14H6O8 | 300.999 | 229.012, 257.008 | 484.07 | [48] |
| 11    | 5.54     | Rutin | Flavonol | C27H30O16 | 609.14 | 271.024, 301.0345, 609.14, 150.99, 300.264 | 610.152 | [49] |
| 12    | 5.621    | Apigenin 7-glucoside | Flavone glucoside | C21H20O10 | 431.09 | 283, 269 | 432.10 | [50,51] |
| 13    | 5.773    | Myricetin 7-rhamnoside | Flavonoid glucoside | C21H20O12 | 463.08 | 301.033, 272.0277, 255.0285 | 564.09 | [52] |
| 14    | 5.903    | Astragalin 7-rhamnoside | Dihydroxyflavone Galactoside | C27H22O12 | 593.14 | 557.24, 284.03, 164.83 | 594.15 | [53] |
| 15    | 6.062    | Melitic acid A | Phenolic acid | C27H22O12 | 583.10 | 431.09, 583.11, 311.0, 169.01 | 538.10 | [50] |
| 16    | 3.935    | Methyltrihydroxy benzoate Phenolic acid | C8H8O5 | 183.02 | 183.02, 124.01, 293.12 | 184.03 | [50] |
| 17    | 7.251    | (8R,8’R)-Secoisolariciresinol 9-glucoside Lignan glucoside | C26H36O11 | 524.22 | 523.21, 300.99, 169.01, 446.97 | 524.225 | [54] |
| Sl. No | Rt (Min) | Identified Compound | Class of Compound | Molecular Formula | [M–H]−/M/Z | MS/MS Fragment Ions | Mass | Reference |
|-------|----------|---------------------|-------------------|------------------|------------|---------------------|------|-----------|
| 1     | 4.288    | 2-O-Ferruloylhydroxycitric acid | Phenolic acid | C16H16O11 | 385.07     | 385.07, 247.02, 468.11, 177.05 | 384.07 | [55] |
| 2     | 5.1      | Kaempferol 7-O-glucoside | Flavanol glucoside | C21H20O11 | 449.10     | 450.11, 299.05, 165.01, 595.16 | 448.10 | [50] |
| 3     | 5.4      | Cynaroside | Glycosylxylflavone | C21H20O11 | 449.10     | 449.11, 329.06, 191.08 | 448.10 | [56] |
| 4     | 5.59     | Cicerin 7-(6-malonyl glucoside) | Isoflavonoid-o-glycoside | C26H26O15 | 601.12     | 564.17, 433.11, 329.06, 300.99 | 578.13 | [57] |
| 5     | 5.59     | 6-C-Fucosyl luteolin | Flavonoid-C-glycosides | C21H20O11 | 433.11     | 433.11, 313.07, 165.01, 235.05 | 432.10 | [58] |
| 6     | 5.652    | Hyperoside | Tetrahydroxyflavone | C21H20O12 | 465.10     | 303.05, 229.04, 433.11 | 464.09 | [50] |
| 7     | 5.685    | Nogalonic acid methyl ester | Dihydroxyanthraquinone | C21H16O18 | 397.09     | 433.11, 323.96, 283.06, 303.05 | 396.08 | [59] |
| 8     | 6.13     | Gambiriin C | Condensed tannin | C30H26O11 | 585.12     | 585.12, 586.12, 313.07, 287.05, 283.06 | 562.13 | [60] |
| 9     | 11.48    | 3-Oxo-12,18-ursadien-28-oic acid | Triterpenoid | C30H44O3 | 452.33     | 224.12 | 452.33 | [48,61] |
| 10    | 6.156    | (3S,4S)-3-Hydroxytetradecane-1,3,4-tricarboxylic acid | Phenolic acid | C17H30O7 | 369.19     | 369.188, 540.24 | 346.20 | Pubchem id: 5460247 |
| 11    | 6.202    | Kaempferol | Tetrahydroxyflavone | C15H10O6 | 287.05     | 287.05, 449.10, 585.12, 586.12 | 286.04 | [49] |
| 12    | 6.541    | Quinidinone | Alkaid | C20H22N2O3 | 323.7      | 192.08, 193.08, 432.10, 323.17, 324.17 | 322.16 | Pubchem id: 84497 |
| 13    | 7.684    | 5,6,7,3′,4′-Pentahydroxyisoflavone | Flavone | C15H10O7 | 303.05     | 303.05, 305.05 | 302.04 | [62] |
| 14    | 7.75     | Maculosidine | Alkaid | C14H13NO4 | 260.09     | 199.06, 227.05, 228.06 | 259.08 | [63] |
| 15    | 26.71    | Anhalonidine | Alkaid | C12H17NO3 | 224.12     | 224.12, 165.005, 222.11, 230.14 | 223.12 | [64] |
| 16    | 4.99     | Hemipic acid | Benzoic acid | C10H10O6 | 249.04     | 249.05, 207.02 | 226.05 | Chemspider id: 61516 |
4. Discussion

In the wake of upsurge in drug resistance, which poses a huge threat to public health across the globe, the lack of alternative treatments has led to a challenge in developing new molecules with antimicrobial properties [65,66]. As an alternative, natural products derived from medicinal plants has been extensively used in combating drug resistance with wide range of biological activities [67].

The present study aims to purify the acetone leaf extract to obtain a biologically active fraction TCAF2, which exhibited noteworthy activity at the lowest concentration against all of the test pathogens, with the lowest concentration being 0.097 mg/mL. The Gram-negative organisms were inhibited at the lowest concentration, which is in contrast with the findings that they are less susceptible to plant extracts because of the cell wall constitution [68]. The results fill the gap that Gram-negative organisms are resistant or less susceptible to plant extracts. Previous research revealing the anti-MRSA activity of an ethanol leaf extract of *T. catappa* with moderate inhibitory activity corroborates the present study [69]. Different leaf extracts of *T. catappa* were active against MRSA, *S. aureus*, and other Gram-negative bacteria, and the MIC was in the range from 25 mg/mL to 3.12 mg/mL [70]. Our study reports the significant inhibition of the test pathogens at a very less concentration in particular reference to MRSA and *S. aureus* (clinical isolate). Other research suggested that the polar extracts such as methanol and ethanol have great inhibitory activity against Gram-positive organisms [71,72], which can be correlated with the present study reporting on the acetone extract, a polar component, exhibiting significant inhibitory activities. The activity of the polar extracts was reported due to the presence of a high level of water-soluble tannins in *T. catappa* which generally exhibit antibacterial activity [73]. The MBC of the test bacteria was slightly higher than the MIC. Significant cidal properties were observed in *S. typhi* (MTCC733) and the clinical isolate *S. aureus* compared with the other test bacteria.

The dermatophytic fungi *M. gypseum*, *M. canis*, and *T. rubrum* exhibited significant inhibition with MIC ranging from 0.195 to 0.097 mg/mL. The fraction also exhibited noteworthy activity against *C. albicans* and *T. asahii*. Fungal skin infections are a significant health concern among which dermatophytosis stands as a significant problem, invading the keratinophilic region in the skin. The demand for potent antifungal agents has demanded for the use of medicinal herbs, which are free from side effects [74]. The current results provide promising results that could serve as lead candidates in treating superficial skin infections caused by the aforementioned dermatophytes. Different extracts of *Terminalia* were investigated for antifungal activity against *C. albicans*, *M. canis*, and *Aspergillus fumigatus*, which reported good antifungal activity [75]. Different species of *Terminalia* and Combretaceae have been reported to possess potent antifungal activity against different species of *Candida* such as *C. krusei*, *Candida glabrata*, *Candida albicans*, and *Candida neoformans*, which exhibited MIC values ranging from 6.20 to 12.25 mg/mL [76]. Other work has reported the antifungal potential of polar extracts *T. catappa* against *M. gypseum* and *M. canis* [77,78]. Botanicals exhibiting MICs in the range of 100–1000 µg/mL are classified as antimicrobials [79]. The result obtained in this study justifies the antimicrobial activity of TCAF2. Time kill studies are important because they provide information about the pharmacodynamics of the antibacterial agent, which indicates the bacteriostatic or bactericidal property at a given concentration and at a particular time point in relation to the growth kinetics of the bacteria [80]. The present study reports the bactericidal and bacteriostatic activity against the test pathogens. Rapid killing was observed at higher concentrations against *S. aureus* (clinical isolate) and *S. typhi* after 2 h of incubation. At smaller concentrations (MIC and four-fold MIC), the killing of bacteria took longer time. This pattern suggests that the bioactivity of the fraction is dependent on the concentration and time of exposure. MRSA 1503 and *Proteus vulgaris* (clinical isolate) exhibited bacteriostatic properties. At higher concentrations, MRSA1503 cells were inhibited significantly after 2 h of exposure, indicating strong bacteriostatic activity in a very short period of time. *Proteus vulgaris* (clinical isolate) exhibited bacteriostatic activity at higher concentrations.
The time taken for the cells to be killed was higher compared with other strains because of the cell wall composition of the bacteria. Higher concentrations of the extracts caused increased diffusion of the bioactive molecules, which causes damage to the cell wall and DNA, enzyme inhibition, and protein synthesis by killing or inhibiting the bacteria [81]. A time kill curve of the fraction was presented for the first time to the best of our knowledge. A few studies are available on the time kill curve for fungi [82], but reports on the bacteria, in particular to clinical isolates and MRSA, are scarce.

Rapid identification and characterization of unknown compounds in the purified fraction from medicinal plants can be attained by analytical technique such as UHPLC–QTOF–MS/MS analysis, which alleviates the speed and selectivity of the analysis and allows for use of the minimal amount of a sample [83]. The TCAF2 analyzed by UHPLC–QTOF–MS/MS was found to be rich in polyphenols, which were previously known to exhibit antimicrobial activity [84–86]. A majority of the phytochemicals identified in the fraction were flavonoid and its glycosides, which are abundantly present in *Terminalia* species [87], which is also in accordance with the total flavonoid estimation and reveals a high flavonoid content. Flavonoids are a large group of naturally occurring compounds with low systemic toxicity and have been extensively studied for their antibacterial activity [88]. Flavonoids such as apigenin, isoflavones, flavone glycosides, quercetin, rutin, and glucosides of luteolin are reported to possess antibacterial activity against different human pathogenic bacteria, which is also evident from the present findings [89–92]. Myricetin inhibited the biofilm formation of *E. coli* at an IC50 concentration of 46.2 µM [93]. Flavonoids including kaempferol, naringin, quercetin, rutin, quinic acid, and luteolin were found to inhibit Gram-positive, Gram-negative, and MRSA strains [94–96]. The present investigation justifies the strong antimicrobial activity of the flavonoids identified in the TCAF2. The antifungal activities of apigenin and rutin were reported against different *Candida* sp. (MIC: 256 µg/mL), *Trichophyton rubrum* and *Trichophyton beigelii* reported MICs of 5 µg/mL [97,98]. The butanol extract of *T. catappa* has reported the presence of ellagic acid; flavonoid C-glycosides; punicalin; and punicalagin, which is reported to have antifungal activity [99]. Among the ellagitannins and gallotannins identified, Punicacort A and Castacrenin D have been reported in the fruit peels and in the bark of *Terminalia catappa*, respectively [45]. However, Punicacort A and Punicacort B have been reported in other species of Combretaceae members such as *Combretum aculeatum* and *Combretum padoides* [76] and in *Terminalia chebula* [100]. The genus *Terminalia* is known to be rich in ellagitannins [101], which have been proven to have antibacterial activity against different bacterial species by disrupting the cell membrane permeability, impairing the oxidative phosphorylation and inhibition of extracellular microbial enzyme synthesis by killing or inhibiting the growth of the bacteria [102–106], which corroborates the results obtained in the present study with significant antimicrobial activity against the clinical isolate bacteria, MRSA strains, and human pathogenic fungi in vitro. The galloyl moieties and the ester linkage between the gallic acid and glucose of gallic acid promotes the antibacterial activity by damaging the bacterial membrane [107]. Alkaloids were identified for the first time in the purified leaf fraction of *T. catappa*. Punicacort A and Punicacort B are known to be rich in ellagitannins [101], which have been proven to have antibacterial activity against different bacterial species by disrupting the cell membrane permeability, impairing the oxidative phosphorylation and inhibition of extracellular microbial enzyme synthesis by killing or inhibiting the growth of the bacteria [102–106], which corroborates the results obtained in the present study with significant antimicrobial activity against the clinical isolate bacteria, MRSA strains, and human pathogenic fungi in vitro. The galloyl moieties and the ester linkage between the gallic acid and glucose of gallic acid promotes the antibacterial activity by damaging the bacterial membrane [107]. Alkaloids were identified for the first time in the leaf fraction of *Terminalia catappa*.Qualitative identification of alkaloids in *T. catappa* has been reported [108,109]. Other Combretaceae members such as *Combretum molle* [110] and *Combretum zenkeri* [111] have been reported for the qualitative presence of alkaloids but the presence of alkaloids in the main genera of *Combretum* and *Terminalia* has yet to be confirmed [75]. Maculosidine (quinoline alkaloid), identified in TCAF2, has been previously reported from some plants of Rutaceae [63,112]. Anhalonidine, a tetrahydroxy isoquinoline, is found in some Cactus species [64]. However, to the best of our knowledge, individual alkaloid compounds have not been identified in *T. catappa*, and our study is the first to report the identification of individual alkaloid compounds in the isolated fraction of *T. catappa*. 
5. Conclusions

There is significant demand for innovative natural chemicals that are effective against infectious bacteria, including multidrug-resistant bacteria and superficial skin infections causing fungi. In this work, we report the promising antibacterial, anti-dermatophytic, and anti-candida activities of TCAF2, rich in polyphenols characterized by the UHPLC–MS/MS technique. The bactericidal properties were exhibited by the fraction because of the presence of a high number of polyphenols. Confirmation of the presence of individual alkaloid compounds in the T. catappa fraction were performed by the UHPLC–MS/MS method for the first time. Further isolation of these alkaloid compounds and other antimicrobial compounds found in this study remains a future scope of this work, which paves way for the development of new antimicrobial therapies through understanding the mechanism of action in treating multidrug-resistant bacteria and skin-infecting dermatophytes. Cytotoxicity assays and in vivo evidence of the bioactivity of the fraction and isolation, and structural elucidation of the compounds lead to the development of new antimicrobial molecules to combat the drug resistance problem.

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