In vitro anticiandidal activity and gas chromatography-mass spectrometry (GC-MS) screening of Vitex agnus-castus leaf extracts

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Background Candida infections are becoming more drug resistant; it is necessary to search for alternative medications to treat them. Therefore, the present study estimates the anticiandidal activity of Vitex agnus-castus (VA-C) leaf extracts. Methods We used the agar well diffusion method to assess the anticiandidal activity of three different VA-C leaf extracts (ethanol, methanol, and water) against three Candida species (Candida tropicalis, Candida albicans, and Candida ciferrii). The minimum inhibitory concentration (MIC) was estimated using the two-fold dilution method and the minimum fungicidal concentration (MFC) was determined using the classic pour plate technique. The MFC/MIC ratio was calculated to estimate the microbicidal or microbiostatic activity. A gas chromatography mass spectrometer was used to screen the phytochemicals of the VA-C leaf extracts (ethanol, methanol, and water). Results All VA-C extracts ethanol, methanol, and water were significantly inhibited the growth of the test Candida species and the inhibition activity depended on the solvent used and the Candida species. The results showed that C. tropicalis was the most highly inhibited by all extracts followed by C. albicans and C. ciferrii. The MIC values were 12.5 µg/ml to 25 µg/ml, and MFC values were 25 µg/ml to 100 µg/ml. The ratios of MFC/MIC were two-fold to four-fold which was considered candidacidal activity. Ninety-five phytochemical compounds were identified by the GC-MS assay for the VA-C leaf extracts. The total number of compounds per extract differed. Methanol had 43 compounds, ethanol had 47 compounds, and water had 52 compounds. The highest compound concentrations were: 4,5-Dichloro-1,3-dioxolan-2-one in ethanol and methanol, 1H-Indene, 2,3-dihydro-1,1,2,3,3-pentamethyl in ethanol, Isobutyl 4-hydroxybenzoate in methanol, and Benzoic acid and 4-hydroxy- in water. These phytochemical compounds belong to different bioactive chemical group such as polyphenols, fatty acids, terpenes, terpenoids, steroids, aldehydes, alcohols, and esters, and most of which have anticiandidal activity. Conclusions VA-C leaf extracts may be useful alternatives to anticiandidal drugs.
based on their effectiveness against all test *Candida* species at low concentrations. However, appropriate toxicology screening should be conducted before use.
In vitro anticandidal activity and gas chromatography-mass spectrometry (GC-MS) screening of *Vitex agnus-castus* leaf extracts

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ABSTRACT

Background

Candida Infections are becoming more drug resistant; it is necessary to search for alternative medications to treat them. Therefore, the present study estimates the anticandidal activity of *Vitex agnus-castus* (VA-C) leaf extracts.

Methods

We used the agar well diffusion method to assess the anticandidal activity of three different VA-C leaf extracts (ethanol, methanol, and water) against three *Candida* species (*Candida tropicalis*, *Candida albicans*, and *Candida ciferrii*). The minimum inhibitory concentration (MIC) was estimated using the two-fold dilution method and the minimum fungicidal concentration (MFC) was determined using the classic pour plate technique. The MFC/MIC ratio was calculated to estimate the microbicidal or microbiostatic activity. A gas chromatography mass spectrometer was used to screen the phytochemicals of the VA-C leaf extracts (ethanol, methanol, and water).

Results
All VA-C extracts ethanol, methanol, and water were significantly inhibited the growth of the test Candida species and the inhibition activity depended on the solvent used and the Candida species. The results showed that C. tropicalis was the most highly inhibited by all extracts followed by C. albicans and C. ciferrii. The MIC values were 12.5 µg/ml to 25 µg/ml, and MFC values were 25 µg/ml to 100 µg/ml. The ratios of MFC/MIC were two-fold to four-fold which was considered candidacidal activity. Ninety-five phytochemical compounds were identified by the GC-MS assay for the VA-C leaf extracts. The total number of compounds per extract differed. Methanol had 43 compounds, ethanol had 47 compounds, and water had 52 compounds. The highest compound concentrations were: 4,5-Dichloro-1,3-dioxolan-2-one in ethanol and methanol, 1H-Indene, 2,3-dihydro-1,1,2,3,3-pentamethyl in ethanol, Isobutyl 4-hydroxybenzoate in methanol, and Benzoic acid and 4-hydroxy- in water. These phytochemical compounds belong to different bioactive chemical group such as polyphenols, fatty acids, terpenes, terpenoids, steroids, aldehydes, alcohols, and esters, and most of which have anticandidal activity.

Conclusions

VA-C leaf extracts may be useful alternatives to anticandidal drugs, based on their effectiveness against all test Candida species at low concentrations. However, appropriate toxicology screening should be conducted before use.

Introduction

The number of severe Candida infections is on the rise, which is concerning due to their virulence, ability to survive in extreme environments, and resistance to antifungal agents (Paramythiotou et al., 2014). Candida species can cause a wide variety of infections ranging from mild to severe, such as candidemia which has a mortality rate up to 38% in immunosuppressed patients (i.e. organ transplantation patients, patients under chemotherapy, HIV- infected, and diabetic) (Koehler et al., 2019; de Oliveira Santos et al., 2018). The rate of fungal infections, including candidiasis, can reach 20% in the intensive care unit and antifungal medications including azoles, echinocandins, fluoropyrimidines, and polyenes are typically used to treat these infections. However, determining the appropriate dose for treatment is challenging when considering their side effects (Chatelon et al., 2019). Candidiasis is one of the most common fungal diseases in the world and includes cutaneous candidiasis, mucosal candidiasis, onychomycosis, and systemic candidiasis. Healthy individuals are also susceptible to candidiasis (de Oliveira Santos et al., 2018). Genus Candida is deuteromycetes fungi and belongs to the Cryptococcaceae family, with up to 200 species. There are thirty species most commonly isolated in human infections including Candida albicans, Candida tropicalis, Candida dubliniensis, Candida parapsilosis, Candida glabrata, Candida lusitaniae, Candida kefyr, and Candida krusei (Rodrigues et.al., 2019; Kim et al., 2016; Brandt and Lockhart, 2012; Miceli et al., 2011).
Antifungals have a broad range of applications but it is difficult to determine the ideal treatment regime because their use can be limited and is often accompanied by side effects. The indiscriminate use of antibiotics has led to an increased resistance to these types of medications (de Oliveira Santos et al., 2018). Accordingly, researchers are exploring therapeutic alternatives, such as the use of plant essential oils or extracts. These have been proven beneficial in the treatment of several diseases due to their phytochemical components that have physiological and therapeutic effects on humans, limited toxicity, and low therapeutic costs (Abdulrasheed et al., 2019; Sardi et al., 2013). The World Health Organization reports indicate that up to 25% of modern medicines used in the United States of America originate from plants. In Africa and Asia, 80% of the population still uses medicinal herbs in their primary health care centers (WHO, 2002). Moreover, there is documented evidence for the antimicrobial potential of more than 1340 plants (Yilar et al., 2016). *Vitex* is one of the largest of the 250 genera in the family Verbenaceae found worldwide (Ganapaty and Vidyadhar, 2005). The therapeutic applications of *Vitex agnus-castus* (VA-C) and its safety as a medicinal plant are well stated (Niroumand et al., 2018; Neves and da Camara, 2016; Rani and Sharma, 2013). Previous studies have emphasized the antibacterial activity of the essential oils extracted from the seeds and fruit of VA-C (Eryigit et al., 2015; Dervish-Shengjergji et al., 2014; Ghannadi et al., 2012). Other studies have investigated the antimicrobial activity of essential oils extracted from the leaves of VA-C (Katirae et al., 2015; Ulukanli et al., 2015). A few studies have demonstrated the antifungal activity of the seed oil (Asdadi et al., 2014). The antifungal potential of VA-C leaves essential oils against plant pathogens (Yilar et al., 2016). The antibacterial activity of the leaf extract of VA-C has been identified in a few studies (Ababutain and Alghamdi, 2018; Kalhoro et al., 2014; Arokiyaraj et al., 2009) as well as the antimicrobial activity of VA-C leaf extract (Kalhoro et al., 2014; Maltaş et al., 2010). These studies used only a few bacteria and one *Candida* species (*Candida albicans*). Keikha et al. (2018) evaluated the antifungal activity of ethanolic and aqueous lead extracts on *C. albicans* strains and they found that the ethanol extract was more effective than the aqueous extract against *C. albicans* strains. However, the effect of VA-C leaf extracts of against human *Candida* species has not been well-studied.

Therefore, this study aims to investigate the anticandidal activity and efficiency of VA-C leaf extracts (water, methanol and ethanol) against the three most frequently isolated *Candida* species (*Candida albicans*, *Candida tropicalis* and *Candida ciferrii*). We determined the phytochemicals of these extracts using Gas Chromatography-Mass Spectrometry (GC-MS).

**Materials & Methods**

**Plant material**

*Vitex agnus-castus* VA-C leaves were collected from a private garden in Dammam City, Saudi Arabia belonging to Ibtisam Mohammed Ababutain. The plant was identified according to Brickell and Zuk (1997).

**Preparation of plant extracts**
VA-C leaves were washed with tap water and left to dry for two days at room temperature in a well-ventilated room using a fan to speed up the drying process, then ground to a fine powder. Maceration method described by Pandey and Tripathi (2014) was used with little modification, in which 60 g of the leaf powder was transferred to three Erlenmeyer flasks containing 300 mL of the three different solvents: distilled water, methanol (80%), and ethanol (80%) to a final concentration of 20% g/mL. The leaf mixtures were shaken for 72 hours at 300 rpm/min/20°C to extract the active compounds. We used the method previously described in Ababutain (2019) to extract the active compounds as follows: the leaf mixtures were filtered twice, first using Whatman No. 1 filter paper and then using bacterial filters. The filtrates were concentrated in an oven at 80°C. The residues were re-suspended in dimethyl sulfoxide (DMSO) to a final concentration of 20%. All flasks were kept at 4°C for further use.

Agar well-diffusion method

Three different prepared VA-C leaf extracts with a 20% (mg/ml) concentration were screened for their anticandidal activity using the agar well-diffusion method (NCCLS, 1993) against three unicellular fungi. Candida tropicalis and Candida albicans were provided by King Fahd Hospital, Al Khobar, Kingdom of Saudi Arabia. Candida ciferrii was obtained from the Biology Department, College of Science, Imam Abdulrahman Bin Faisal University.

Inoculums of the Candida species were prepared from new cultures in potato dextrose broth (PDB). A Biomerieux DensiCHEK plus meter device was used to adjust the cell suspension turbidity at 1-2 x 10^6 CFU/ml, which represents 0.5 McFarland standards. Each Petri dish was inoculated individually with 0.5 ml of the previous suspension. Melted potato dextrose agar (PDA) was poured over the inoculums, and the plates were rotated to ensure even distribution of the inoculums then left to harden at room temperate for 5 min. Five wells were made on the inoculated PDA using a 6 mm sterile cork-borer. Each well was filled with 100 µL of the plant extracts. Positive and negative controls were included; nystatin (10 mcg) was used as the positive control and DMSO was used as the negative control. The plates were incubated at 37°C for 24 hours. The anticandidal activity of the plant extracts was estimated in millimeters (mm) using a ruler and measuring the free growth zones around the wells. The experiments were performed in three replicates to ensure the reliability of the results.

Determination of minimum inhibitory concentration (MIC)
The minimum inhibitory concentration (MIC) of VA-C leaf extracts was estimated using the two-fold dilution method (Omura et al., 1993) as well as the method previously described in Ababutain (2019). Briefly, the plant extracts were diluted with PDB media using 96-well microtiter plates in wells 1 to 10. Standard Candida inoculums at a concentration of 1-2x10^6 CFU/mL were transferred to the wells to make a final concentration of 50%. We used growth media with the Candida inoculum in well 11 and growth media with plant extracts in well 12, as positive and negative controls, respectively. The turbidity was examined by the naked eye after an overnight incubation period at 37°C and the lowest concentration of plant extract showing no Candida species growth was recorded as MICs. All experiments were performed in three replicates.

Determination of minimum fungicidal concentration (MFC)

The classic pour plate technique was used to determine the MFC (NCCLS, 1997). Concentrations that showed no Candida species growth from previous MIC experiments were transferred to Petri dishes, then 15 mL of melted PDA was poured over it and gently rotated and left to solidify. Inoculated Petri dishes were incubated at 37°C for 48 hours. The lowest concentration that showed no visible Candida species colonies were recorded as MFC (Ababutain, 2019). All experiments were performed in three replicates.

Determination of anticandidal efficiency

The anticandidal efficiency of VA-C leaf extracts (ethanol, methanol and water) was determined by calculating the ratio of MFC/MIC according to Levison and Levison (2009).

Gas chromatography-mass spectrometry (GC-MS)

We analyzed the bioactive compounds of all three VA-C leaf extracts (ethanol, methanol and water) with a gas chromatography-mass spectrometer (Shimadzu-Japan) model QP2010 SE, with a 5 Sil MS 5% diphenyl/ 95% dimethyl polysiloxane capillary column (0.25-μm df, 30 meter, 0.25 mmID) using the method previously described in Ababutain (2019). One microliter from each diluted plant extract (100/1400, V/V in DMSO) was injected individually in the split mode with a split ratio of 1:10. We used the electron impact ionization system at 70eV ionization energy to determine GC-MS exposure or detection. Pure helium (99.999%) was used as a carrier gas, at a constant column flow 0.7ml/ min and total flow of 10.4 ml/min. The flow control mode had a linear velocity of 29.6cm/sec. The injector temperature was set at 250°C and the ion-source temperature was set at 250°C. The column temperature was programmed at 50°C to 300°C, with a hold time of 3 min, and a total run time of 29 min. The chemical compounds were identified using the National Institute of Standards and Technology (NIST 08) library match and the quantitative data were generated automatically as a percentage (Adams, 2007).

Statistical analysis
The anticandidal activity of the VA-C leaf extract between the solvents and the Candida species was conducted using one-way Anova test. A P-value of <0.01 was considered statistically significant. Statistical data were analyzed using Statistical Packages for Software Sciences (SPSS, 2013) version 21 Armonk, New York, IBM Corporation.

Results

Anticandidal activity of VA-C leaf extracts

The VA-C extracts were shown to inhibit the growth of all tested Candida species and the inhibition activity depended on the solvent type and Candida species. The results showed that C. tropicalis was the most inhibited by all the extracts followed by C. albicans and C. ciferrii (all P=0.01). The effects of the ethanol extract against C. tropicalis, C. albicans and C. ciferrii were significantly higher compared to water and methanol extracts at P=0.01, P=0.037 and P=0.047, respectively (Table 1).

MIC results were between 12.5 µg/ml to 25 µg/ml and all extracts showed similar activity against all Candida species at MIC 25 µg/ml, except C. tropicalis which was the most sensitive to the ethanol extract at MIC 12.5 µg/ml. The MFC results were between 25 µg/ml to 100 µg/ml. Most extracts showed similar MFC values against all Candida species at MFC 50 µg/ml except C. tropicalis. The MFC ethanol extract at 25 µg/ml had the highest anticandidal activity against C. tropicalis and the MFC methanol extract at 100 µg/ml was considered to be the lowest anticandidal activity against C. albicans. The results revealed that both MIC and MFC values for all three solvents were narrow where the differences between values were one to two concentrations only. The MFC/MIC ratio in all the three extracts were only two-fold to four-fold, which means that VA-C leaf extracts are potentially candidacidal (Table 2).

Gas chromatography -mass spectrometry (GC-MS) analysis

Our results revealed that VA-C leaf extracts are rich in phytochemical components of different concentrations. 95 chemical compounds were extracted depending on the solvent type and, of these, 13 compounds were extracted by all three solvents and the total number of extracted compounds was 52 by water extraction, 47 by ethanol extraction, and 43 by methanol extraction (Table 3).

Discussion
Antibiotic resistance is becoming more common among a larger number of microorganisms, including *Candida* species, leading to a heightened interest in finding alternative treatments. The secondary metabolites of plants have made them useful for treating a variety of diseases, flavoring foods and products, preserving food, in pesticides, in perfumes and cosmetics, and more recently to inhibit the microbial growth. VA-C leaf extracts have been reported to cause mild and reversible side effects such as headache, acne, nausea, gastrointestinal disturbances, erythematous rash, pruritus, and menstrual disorders. However, no drug interactions have been associated with VA-C leaf extracts (Daniele et al., 2005). Therefore, VA-C leaf extracts (ethanol, methanol, and water) were investigated for their ability to inhibit the growth of three Azoles antibiotic-resistant *Candida* species: *C. ciferrii*, *C. albicans*, and *C. tropicalis* (Romald et al., 2019; Bhakshu, et al., 2016).

Our results showed that alcohol extracts (methanol and ethanol) and aqueous extract have the ability to inhibit the growth of all tested *Candida* species. These results are in agreement with Kalhora et al.’s study (2014), which found that the ethanol VA-C leaf extract has the potential to inhibit the growth of *C. albicans*. Our results are also consistent with Maltaş et al., (2010) who observed that the methanol extract of VA-C leaves inhibits the growth of *C. albicans*. Moreover, we showed that the inhibitory capacity of the solvents varied significantly in descending order of ethanol, then water, then methanol. These results are in line with a recent study conducted by Keikha et al. (2018) who found that VA-C ethanol leaf extract has the highest inhibiting effect against *C. albicans* isolates than water extract. Our results showed a similarity in the inhibitory effect of all extracts with nystatin (10mcg) as a positive control against *C. albicans*, where the inhibitory effect for the positive control was higher than all extracts against *C. tropicalis*.

We found that MIC showed that the ethanol extracts of VA-C were relatively higher than water and methanol. MIC values were between 12.5 µg/ml and 25 µg/ml for ethanol and represented a dilution of 4 and 3, respectively. For water and methanol the MIC values are specified at 25 µg/ml which represents dilution 3. Our results are similar to those of Keikha et al. (2018) who also found that the ethanol extract of VA-C was more effective than the aqueous extract when the MIC values of ethanol against isolates of *Candida* species were between 0.78 µg/ml and 1.56 µg/ml, which represent dilution 7 and 8, respectively. The values of the aqueous extract were between 6.25 µg/ml and 1.562 µg/ml, which represent dilutions of 5 and 7, respectively.

There was a convergence of MFC values, which represents only the three dilutions from 1 to 3 (100 µg/ml and 25 µg/ml), respectively. The VA-C extract of ethanol was the most influential on *C. tropicalis* with the value of MFC 25 µg/ml and the aqueous extract was less effective on *C. albicans*, with a value of 100 µg/ml.
Selection of antibiotics for the treatment of infections is highly influenced by the mechanism of action. Antibiotics classified into either by killing the microbe (microbicidal) or inhibiting its growth (microbistatic) (Etebu and Arikekpar, 2016). Antibiotics with inhibitory effects are usually prescribed to patients who do not have problems with their immune system, while antibiotics with a fatal effect are prescribed for patients with low immunity or severe infections (Davies and Davies, 2010). Candida species are generally opportunistic and affect the group of people with low immunity so antibiotics that are prescribed are generally more effective if they are of the fatal type. Therefore, the inhibitory efficiency of the VA-C extract was estimated using the ratio between MFC and MIC. Our results showed that the ratio of MFC/MIC between two-fold to four-fold have a candidacidal effect (Levison and Levison, 2009). To our best of our knowledge, ours is the first study to establish this finding.

We found that the extracts of VA-C differed in their inhibitory effect according to the type of solvent and this is may be due to the difference in the degree of polarity between the solvent. Water has the highest polarity of 1,000 followed by methanol (0.762) and finally, ethanol (0.654). The compounds extracted by these highly polar solvents differ in quantity and quality (Abubakar and Haque, 2020). Many studies have demonstrated the effect of the solvent type on the inhibitory potential of plant extracts (Aljuraifani 2017; Ababutain 2015).

The GC-MC analysis result revealed that all three VA-C extracts were rich in chemical compounds that act as an anti-inflammatory, anticancer, anti-Alzheimer, anti-diarrheal, anti-diabetic, anti-viral, antioxidant, anti-allergic, nematicide, antibacterial, antifungal. These extracts are also used as food preservatives and flavorings, as previously found in other published works (Table 3). Several of these secondary metabolites belong to important chemical groups such as polyphenols, fatty acids, terpenes, terpenoid, steroids, aldehydes, alcohol, and esters. These results are in agreement with a previous study of Keikha et al. (2018), which stated that the VA-C extract was rich in chemical compounds, and the alcoholic extract contained 36 chemical compounds that belong to different chemical groups. Our results showed that the majority of compounds were 4,5-Dichloro-1,3-dioxolan-2-one in both ethanol and methanol, 1H-Indene, 2,3-dihydro-1,1,2,3,3-pentamethyl in ethanol, Isobutyl 4-hydroxybenzoate in methanol, and Benzoic acid and 4-hydroxy- in water. Keikha et al. (2018) found that the majority of compounds in the VA-C ethanol extract were α-Pinene, isoterpinolene, caryophyllene, and azulene. The difference in the number of phytochemical compounds may be attributed to the variations among the VA-C genotypes (Karaguzel and Girmen, 2009).
The inhibitory activity of VA-C extracts maybe attributed to the presence of important bioactive compounds (Abdal Sahib et al., 2019), which may target different structures of the Candida species including the cell wall, cell membrane, and mitochondria enzymes. Some of these compounds may reduce or prevent the virulence factors, including adhesins, enzymes production, germ tubes (Pseudohyphal), biofilm formation, and quorum sensing (de Oliveira Santos et al., 2018; Liu et al., 2017, Sardi et al., 2013). Our results showed the diversity of the compounds extracted from VA-C plant leaves that belong to several effective biochemical compounds with different anticandidal activity, including polyphenols that can destroy the Candida cell membrane leading to permeability of the cell contents (Peralta et al., 2015; Hwang et al., 2011; Hwang et al., 2010), inhibition of mitochondrial enzyme activity in the Candida cell (Yang et al., 2014) and inhibition of the germ tube formation (Seleem et al., 2016). Fatty acids with carbon chains between 10-12 carbons had a good inhibitory effect against Candida species (Ababutain, 2019; Bergsson et al., 2001). Terpenes have been reported to have inhibitory effects against C. albicans and may prevent biofilm formation (Pemmaraju et al., 2013). Terpenoids inhibit C. albicans cell growth by affecting the membrane and preventing adhesins, biofilm formation, and germ tube formation (Touil et al., 2020; Raut et al., 2013; Zore et al., 2011).

Conclusions

Our results showed that VA-C leaf extract is rich in bioactive compounds with broad spectrum activity that inhibited all the tested Candida species despite different species. Accordingly, VA-C leaf extracts may inhibit the growth of Candida species in general, compared to antifungals that affect a specific species or a strain of species and require an accurate diagnosis of the Candida isolation to choose the appropriate antifungal. The inhibitory activity of the ethanol solvent was better than methanol and water, which may indicate the importance of choosing the appropriate solvent to extract phytochemicals with high inhibiting effectiveness and in higher quantities. Moreover, our results showed that the extract had a candidacidal effect on test Candida species at low concentrations, which may reduce the side effects of the extract. VA-C leaf extracts are advantageous, and a promising component that can be used to develop an alternative anticandidal agent. Further studies are required to assess the toxicity, genotoxicity and mutagenicity of VA-C extracts and prove their safety for human use.

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Table 1. Anticandidal activity of VAC leaves extract at concentration of 20% by using well diffusion assay

*P-value has been calculated using one-way Anova. ** Significant at p<0.01 level. ND, not identified
| Candida species | Nystatin (10 mcg) | negative control | Ethanol | Water | Methanol | P-value * |
|-----------------|-------------------|-----------------|---------|-------|----------|----------|
| C. tropicalis   | 11.0 ± 1.00       | 0               | 7.50 ± 0.50 | 5.67 ± 0.29 | 5.33 ± 0.29 | 0.01 ** |
| C. albicans     | 5.83 ± 0.29       | 0               | 5.83 ± 0.29 | 5.00 ± 0.50 | 5.00 ± 0.50 | 0.047 ** |
| C. ciferrii     | ND                | 0               | 4.33 ± 0.58 | 3.33 ± 0.29 | 3.33 ± 0.29 | 0.037 ** |
| P-value         | 0.01 **           | -               | 0.01 ** | 0.01 ** | 0.01 ** | --       |
Table 2. Minimal Inhibitory Concentration (MIC) µg/ml and Minimal Fungicidal Concentration (MFC) µg/ml and their ratio of VA-C leaves extracts.

*Ratio MFC/MIC
| Candida species | Ethanol | Water | Methanol |
|-----------------|---------|-------|----------|
|                 | MIC     | MFC   | Ratio*   | MIC | MFC | Ratio* | MIC | MFC | Ratio* |
| *C. tropicalis* | 12.5    | 25    | 2        | 25  | 50  | 2      | 25  | 50  | 2      |
| *C. albicans*   | 25      | 50    | 2        | 25  | 50  | 2      | 25  | 100 | 4      |
| *C. ciferrii*   | 25      | 50    | 2        | 25  | 50  | 2      | 25  | 50  | 2      |
Table 3. GC-MS analysis of VA-C leaves extracts, their molecular formula, nature and biological activities.
| No | Compound name                                                                 | Peak Area% | Molecular Formula | Compound nature and biological activities                                      |
|----|--------------------------------------------------------------------------------|------------|-------------------|--------------------------------------------------------------------------------|
| 1  | 4,5-Dichloro-1,3-dioxolan-2-one                                                 | 7.43       | C₆H₅ClO₂         | No report was found.                                                            |
| 2  | Benzoic acid, 4-hydroxy-                                                       | 2.13       | C₇H₆O₃           | Phenolic compounds (Eseyin et al., 2018).                                       |
| 3  | 5-Hydroxymethylfurfural                                                        | 1.18       | C₆H₄O₃           | Organic compound Antioxidant and Antiproliferative (Ibrahim et al., 2016).      |
| 4  | Phenol                                                                         | 1.12       | C₆H₅OH           | Phenolic compound, antiviral, antibacterial and antifungal activities (Özçelik et al., 2011). |
| 5  | 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-                                     | 0.75       | C₆H₄O₄           | Flavonoids, Anti-inflammatory, analgesic, antimicrobial activity (Neeraj et al., 2019). |
| 6  | Catechol                                                                       | 0.50       | C₆H₄(OH)₂        | Polyhydric phenol, antiviral, antimicrobial activities (Özçelik et al., 2011). |
| 7  | Benzeneacetaldehyde, alpha-methyl-                                             | 0.35       | C₉H₁₀O           | Hydrotropic aldehyde.                                                           |
| 8  | Benzeneacetic acid, 4-hydroxy3-methoxy,                                        | 0.39       | C₁₀H₁₂O₄         | No report was found.                                                            |
| 9  | Pentanal                                                                       | 0.10       | C₅H₁₀O           | alky aldehyde, Inhibition bacteria (Lamba, 2007).                               |
| 10 | Squalene                                                                       | 0.13       | C₃₀H₄₀            | Terpenoid, Anticandidal activity, antioxidant, anti-inflammatory, and anticancer agent (Ghimire et al., 2016; Zore et al., 2011). |
| 11 | Maltol                                                                         | 0.06       | C₆H₆O₃           | Antimicrobial activity (Saud et al., 2019).                                     |
| 12 | 1H-Benzocyclohepten-7-ol,2,3,4,4a,5,6,7,8-                                     | 0.36       | C₁₅H₂₆O          | Sesquiterpenids (Solaki et al., 2018).                                           |
| 13 | n-Hexadecanoic acid                                                            | 0.70       | C₁₆H₃₂O₂         | Palmitic saturated Fatty acid ester, antimicrobial, antitumor activities, antioxidant, pesticide, nematicide, antiandrogenic and hypcholesteroleni (Tyagi and Agarwal., 2017; Karthikeyan et al., 2014; Sermakkani and Thangapandian, 2012). |
| 14 | 3,5-Octadienoic acid, 7-hydroxy-2-methyl                                       | 0.85       | C₉H₁₄O₃          | No report was found.                                                            |
| 15 | Eugenol                                                                        | 0.39       | C₁₀H₁₂O₂         | Phenolic compounds, antimicrobial activity, insecticide nematicide and food additive (Tan and Nishida, 2012; Johny.
| No. | Name                                                                 | E | R | CAS     | Description                                                                                                                                                                                                 |
|-----|----------------------------------------------------------------------|---|---|---------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 16  | 1,2,3-Benzenetriol                                                   | 0.56 | 0.67 | C₆H₆O₃ | No report was found.                                                                                                                                                                                         |
| 17  | Propylphosphonic acid, di(2-ethylhexyl) ester                        | 2.57 | 1.18 | C₂₁H₄₀O₄ | Antimicrobial activity, food preservative, added to cosmetic products, and pharmaceutical products (Mincea et al., 2009).                                                                                 |
| 18  | Methylparaben                                                        | 0.39 | 0.28 | C₈H₈O₃  |                                                                                                                                                                                                             |
| 19  | 1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene    | 1.21 | 1.04 | C₁₄H₂₂O  | No report was found.                                                                                                                                                                                        |
| 20  | Triacetin                                                            | 0.19 | 0.27 | C₉H₁₆O₆  | Triester of glycerin and acetic acid.                                                                                                                                                                       |
| 21  | 5-(1-Isopropenyl-4,5-dimethylbicyclo[4.3.0]                           | 1.13 | 0.87 | C₂₂H₃₆O₂ | No report was found.                                                                                                                                                                                        |
| 22  | 2,4-Cholestadien-1-one                                              | 1.72 | 0.96 | C₂₃H₄₂O  | No report was found.                                                                                                                                                                                        |
| 23  | Phytol                                                               | 3.31 | 1.81 | C₂₀H₄₀O  | Diterpene, antiviral and antimicrobial activities (Özçelik et al., 2011).                                                                                                                                   |
| 24  | 9,12,15-Octadecatrienoic acid, (Z, Z,Z)-                             | 1.18 | 0.90 | C₁₈H₃₀O₂ | Linolenic Omega-3 polyunsaturated fatty acid, anti–inflammatory (Sermakkani and Thangapandian, 2012).                                                                                                      |
| 25  | Cedran-diol, (8S,14)-                                                | 0.13 | 0.52 | C₁₉H₃₂O₂ | No report was found.                                                                                                                                                                                        |
| 26  | 3,7,11,15-Tetramethyl-2-hexadecen-1-ol                               | 1.11 | 1.34 | C₂₀H₄₀O  | Terpene Alcohol, antioxidant, anti–inflammatory and flavoring agent (Shibula and Velavan, 2015; Jegadeeswari et al., 2012; Sermakkani and Thangapandian, 2012). |
| 27  | Spiro[4.5] dec-9-en-1-ol,1,6,6,10-tetramethyl                        | 0.54 | 0.39 | C₁₄H₂₄O  | No report was found.                                                                                                                                                                                        |
| 28  | Dodeca-1,6-dien-12-ol,6,10-dimethyl                                 | 1.57 | 1.57 | C₁₄H₂₅O  | No report was found.                                                                                                                                                                                        |
| 29  | Octadecanoic acid                                                   | 0.51 | -    | C₁₈H₃₅CO₂H | Stearic saturated fatty acid.                                                                                                                                                                               |
| 30  | Benzenediazonium, 2-hydroxy-, hydroxide, i                          | 0.86 | -    | C₆H₅N₂O  | No report was found.                                                                                                                                                                                        |
| 31  | Vitamin E                                                            | -    | 0.39 | C₂₀H₅₀O₂ | Lipid, antibacterial, anti–alzheimer, antiaging and antioxidant (Kumaravel et al., 2017; Al-Marzoqi et al., 2016; Shahina et al., 2016; Al-Salih et al., 2013). |
|   | Chemical Name            | C, H, O, or component | C, H, O, or component | C, H, O, or component | Molecular Formula | Description                                                                                     |
|---|-------------------------|----------------------|----------------------|----------------------|------------------|-----------------------------------------------------------------------------------------------|
| 32| Cedrol                  | - 0.22 0.12          | C₁₄H₂₆O                | sesquiterpene alcohol. |
| 33| gamma-Sitosterol        | - 0.89 0.56          | C₂₀H₅₀O                | Steroid, antidiabetic drug (Tripathi et al., 2013). |
| 34| Paromomycin             | - 0.12 0.22          | C₂₃H₄₅N₅O₁₄            | Treatment of diarrhea and protozoa infections (Olajuyigbe et al., 2018). |
| 35| Heptanal                | 0.20 - -              | C₇H₁₄O                  | aldehyde antibacterial activity (Lamba, 2007). |
| 36| Ionone                  | 0.33 - -              | C₁₃H₂₆O                | Sesquiterpenoids, antimicrobial agents ( Sharma et al., 2012). |
| 37| Chloroxylol             | 0.16 - -              | C₈H₅OCl                 | phenols with antiseptic activity, It is used in the manufacture of disinfectants and sterilizers (McDonnell, 2009). |
| 38| 1-Heptadecene           | 0.22 - -              | C₁₇H₃₄                  | unsaturated aliphatic hydrocarbons. |
| 39| Undecanal               | 0.25 - -              | C₁₆H₃₁CHO               | fatty aldehyde lipid molecule. |
| 40| 1H-Indene, 2,3-dihydro-1,1,2,3,3-pentamethyl | 9.63 - - | C₁₄H₂₀ | No report was found. |
| 41| Epiglobulol             | 0.97 - -              | C₁₈H₂₆O                | Alcohol. |
| 42| tau-Cadinol             | 1.64 - -              | C₁₃H₂₆O                | No report was found. |
| 43| alpha-Cadinol           | 0.38 - -              | C₁₃H₂₆O                | Antifungal activity (Cheng et al., 2012). |
| 44| Phytol, acetate         | 0.37 - -              | C₂₃H₄₂O₂                | Food additive, antimicrobial, anti-inflammatory, anticancer and antiuretic properties (Sermakkani and Thangapandian, 2012). |
| 45| 1S,2S,5R-1,4,4-Trimethyltricyclo[6.3.1.0(2,5)]1.26 - - | C₁₃H₂₄ | No report was found. |
| 46| beta-iso-Methyl ionone  | 0.39 - -              | C₁₄H₂₂O                | No report was found. |
| 47| Longipinane, (E)-       | 0.41 - -              | C₁₃H₂₄                | No report was found. |
| 48| (-)-Isolongifolol, methyl ether | 0.70 - - | C₁₆H₂₅O | Ether. |
| 49| Taraxasterol            | 0.07 - -              | C₃₀H₅₀O                | Anti-tumor and chemopreventive activity (Ovesná and Horvathova, 2004). |
| 50| S-Methyl methanethiosulphonate | 0.07 - - | CH₃SO₂SCH₃ | Ester, Antimutagentic agent and antimicrobial activity (Joller et al., 2020; Miguel et al., 2016). |
| 51| 1-Heptatriacotanol      | 0.23 - -              | C₁₃H₂₆O                | Fatty alcohol. |
| 52| 2-Vinylfuran            | 0.83 -               | C₆H₆O                  | Antimicrobial activity (Drobnica and Sturidik, 1980). |
| 53| Salicyl hydrazide       | - 0.39 -              | C₇H₈N₂O₂                | Phenolic compounds, antimicrobial activity, Anti-inflammatory (Madan et al., |
| No. | Compound                                                   | pIC (or pK) | LogP | Molecular Formula | Additional Information                                                                 |
|-----|------------------------------------------------------------|-------------|-------|-------------------|----------------------------------------------------------------------------------------|
| 54  | Isobutyl 4-hydroxybenzoate                                 | -           | 8.91  | C₁₁H₁₄O₃          | No report was found.                                                                    |
| 55  | Methyl(ethenyl)bis(but-3-en-1-ynyl) silane                 | -           | 1.62  | C₇H₁₆Si           | No report was found.                                                                    |
| 56  | Beta Carotene                                              | -           | 0.16  | C₄₀H₅₆            | Carotenoids used as food, nutrition, antioxidant, disease control, and antimicrobial agents (Kirti et al., 2014). |
| 57  | 17-Norkaur-15-ene, 13-methyl-(8, beta., 13, b)              | -           | 1.05  | C₂₀H₃₂            | No report was found.                                                                    |
| 58  | 3-Hydroxy-2-(2-methylyclohex-1-enyl) propanoic acid         | -           | 1.48  | C₁₆H₁₆O₂          | No report was found.                                                                    |
| 59  | Cyclopropanebutanoic acid, 2-[2-[2-[2-[2-[(2-pen         | -           | 1.20  | C₁₁H₂₂N₂O₄        | No report was found.                                                                    |
| 60  | Cholan-24-oic acid, methyl ester, (5.beta.)                 | -           | 1.56  | C₂₂H₃₀O₃          | No report was found.                                                                    |
| 61  | Lup-20(29)-en-3-ol, acetate, (3.beta.)                     | -           | 1.12  | C₃₂H₃₂O₂          | No report was found.                                                                    |
| 62  | Geranyl-alpha-terpinene                                     | -           | 0.80  | C₂₀H₃₂            | Terpinene.                                                                             |
| 63  | Tungsten, tricarbonyl-(2,5-norbornadiene)                  | -           | 1.32  | C₁₄H₁₆            | No report was found.                                                                    |
| 64  | 1,2-Cyclopentanediene                                      | -           | 1.21  | C₇H₁₀O₂           | Prevents gastrointestinal tumor growth (Neeraj et al., 2019).                           |
| 65  | 2-Cyclopenten-1-one, 2-hydroxy-3-methyl-                   | -           | 0.34  | C₆H₅O₂            | No report was found.                                                                    |
| 66  | 1,2,3-Propanetriol, 1-acetate                              | -           | 1.23  | C₄H₁₀O₄           | No report was found.                                                                    |
| 67  | Acetoacetic acid, 3-thio-, benzyl ester                    | -           | 0.16  | C₁₁H₁₂O₂          | No report was found.                                                                    |
| 68  | trans-Z-alpha-Bisabolene epoxide                           | -           | 1.11  | C₁₅H₂₄O           | No report was found.                                                                    |
| 69  | 2-Hydroxyoctanoic acid                                     | -           | 0.62  | C₈H₁₆O₃           | No report was found.                                                                    |
| 70  | 1-Tetradecene                                              | -           | 0.67  | C₁₄H₂₈            | Antimicrobial activity (Naragani et al., 2016).                                         |
| 71  | Benzoic acid, 4-methoxy                                   | -           | 0.69  | C₈H₅O₃            | No report was found.                                                                    |
| 72  | Chlorozotocin                                              | -           | 0.20  | C₉H₁₆ClN₃O₇       | No report was found.                                                                    |
| 73  | 2-Isopropyl-5-methyl-6-oxabicyclo [3.1.0] hex              | -           | 1.51  | C₁₀H₁₆O₂          | No report was found.                                                                    |
| 74  | Quinic acid                                                | -           | 2.96  | C₇H₁₂O₆           | Anti-viral activity (Özçelik et al., 2020).                                              |
| No. | Chemical Name | Molecular Formula | Molecular Weight | CAS Number | Note |
|-----|---------------|-------------------|------------------|------------|------|
| 75  | 3-Methylindene-2-carboxylic acid | - - | 1.11 | C_{11}H_{16}O_{2} | No report was found. |
| 76  | O, O-Dibutyl S-(2-acetamidoethylmercapto)p | - - | 1.32 | C_{12}H_{22}O_{4} | No report was found. |
| 77  | 3-Deoxy-d-mannonic acid | - - | 1.21 | C_{6}H_{12}O_{6} | No report was found. |
| 78  | Cyclooctane-1,4-diol, cis | - - | 0.44 | C_{8}H_{16}O_{2} | No report was found. |
| 79  | cis, cis, cis-7,10,13-Hexadecatrienal | - - | 0.58 | C_{16}H_{26}O | Unsaturated fatty aldehyde. |
| 80  | 5-Iodo-7-oxa-2-thia-tricyclo[4.3.1.0(3,8)]de | - - | 1.08 | C_{8}H_{11}I_{2}O | No report was found. |
| 81  | Bicyclo [6.1.0] nonane, 9-(1-methyllethylidene) | - - | 3.66 | C_{12}H_{20} | No report was found. |
| 82  | Inositol | - - | 0.15 | C_{6}H_{12}O_{6} | Essential nutrient, Cancer chemoprevention agent, treatment for Polycystic Ovary Syndrome and insulin sensitizing agent (Carlomagno and Unfer, 2011). |
| 83  | Xylose | - - | 0.15 | C_{5}H_{10}O_{5} | Pentose sugar (Huntley and Patience, 2018). |
| 84  | Scyollo-Inositol | - - | 1.18 | C_{6}H_{12}O_{6} | treatment of Alzheimer's disease (Ma et al., 2012). |
| 85  | 2,4-Pentadien-1-ol, 3-pentyl-, (2Z) | - - | 0.94 | C_{10}H_{18}O | No report was found. |
| 86  | Widdrol hydroxyether | - - | 0.23 | C_{13}H_{26}O_{2} | No report was found. |
| 87  | Stigmasterol | - - | 0.21 | C_{29}H_{48}O | Steroid, antioxidant, antimicrobial, anticancer, antiarthritic, antiasthma, anti-inflammatory, diuretic (Tyagi and Agarwal, 2017; Kumar et al., 2014). |
| 88  | beta.-Amyrin | - - | 0.43 | C_{30}H_{50}O | Triterpenes, anti-inflammatory (Okoye et al., 2014). |
| 89  | 5,5'-Dihydroxy-3,3'-dimethyl-2,2'-binaphthal | - - | 1.31 | C_{17}H_{14}O_{6} | No report was found. |
| 90  | Lanosterol | - - | 0.22 | C_{30}H_{50}O | Sterol, essential components of eukaryotic cells (Wei et al., 2016). |
| 91  | Betulin | - - | 0.58 | C_{30}H_{50}O_{2} | Anti-Viral and anti-tumour (Tolstikov et al., 2005). |
| 92  | alpha-Tocopheryl acetate | - - | 0.23 | C_{31}H_{52}O_{3} | Antimicrobial activity (Bidossi et al., 2017). |
| 93  | Geldanamycin | - - | 0.33 | C_{29}H_{40}N_{2}O_{9} | Chemotherapeutic agents |
|   | Compound     | Retention Time | Mass | Molecular Formula | Note                                                        |
|---|--------------|----------------|------|-------------------|-------------------------------------------------------------|
| 94| Dihydrosteviobiside | -              | 0.26 | C32H52O13         | No report was found.                                        |
| 95| Bronopol     | -              | 0.10 | C3H6BrNO4         | Antimicrobial activity (Birkbeck et al., 2006; Treasurer et al., 2005). |

Total compounds for each solvent: 47, 43, 52