Original article

Inhibition realization of multidrug resistant bacterial and fungal isolates using Coccinia indica extracts

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The crude aqueous and ethanolic leaf extracts of Coccinia indica were screened for methicillin resistant Staphylococcus aureus (MRSA), multidrug resistant (MDR) Streptococcus pyogenes, Escherichia coli, Candida auris and Trichophyton rubrum. Antibacterial and antifungal activities were assessed by standard disc diffusion and tube dilution methods. The results showed that ethanolic extract inhibited MRSA, C. auris at 250 μg/mL and S. pyogenes at 200 μg/mL comparable to the susceptible antibiotics used as positive controls. There was no observable activity against T. rubrum, while a mild activity was observed with ethanolic extracts over E. coli at higher concentrations which did not turn out to be complete or significant inhibition. Aqueous extract did not exhibit any observable activity over the five organisms tested. Furthermore, the results showed clear cut concentration dependent antibacterial and antifungal activities with additional variation of specific activity over Gram positive and negative bacteria, yeast and filamentous fungi. So, it is evident that ethanolic extract of Coccinia indica could be further escalating for mechanistic studies in the era of multidrug resistance, indigenous preparations from herbs could be a safe choice over clinically challenging organisms.

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1. Introduction

Multidrug resistance is a huge problem across globe irrespective of developed or developing world (Aslam et al., 2018). Currently drug resistance forms the basis of the pharmaceutical research especially in the area of drug discovery (Simpkin et al., 2017). Antibiotic and antifungal drug resistant organisms and search for proper drugs to combat them have been always priori-
preparations against multi drug organisms. Though there are many reports on the ingredients of the plant or part of the plant used, we have commonly accepted the formula of traditional formulations in modern medical applications.

In the current investigation, a fast-growing tropical vine Coccinia indica also known as ivy gourd or baby watermelon was selected on the basis of its profound biological and pharmacological activities observed in the literature (Niazi et al., 2009; Raje et al., 2013). Though there some studies pertaining to its antibacterial (Devi et al., 2021) and antifungal activities however, with different species (Venkateswaran and Pari, 2003) almost all parts of the plants have been used as drug in traditional medical branches of Ayurveda and Unani. Many diseases like leprosy, tumor and jaundice have been treated with extract of fruits and leaves due to its antioxidant nature while, animal studies have shown to reduce plasma glucose levels in diabetes (Shifali et al., 2021). Further, dried flower and leaf of the plant is used to treat variety of clinical conditions like eye irritations, skin eruptions, burns, nausea and earache (Ramachandran et al., 2014). There are a few experimental works to demonstrate the antiviral and antihelminthic activity (Arbab et al., 2017). The insulin stimulatory effect and antioxidant properties of the leaf extract was proved recently (Mukherjee et al., 1972).

Therefore, with all these available background studies, it was decided to assess the antibacterial and antifungal activity of the leaf extracts against MDR clinical isolates. To add the reason behind use of the whole extract was authentication of complete classical extracts used in the traditional medical practices.

2. Materials and methods

2.1. Preparation of aqueous and ethanolic extracts

Fresh leaves of Coccinia indica (Cucurbitaceae) were collected from plantations in the south western Saudi Arabia. The leaves were cleaned, dried in shade, crushed to get powder form using a blender (at low-speed) and stored in air tight opaque containers till further extracted. About 100 g of the dried powdered leaves were mixed with 100 mL water and another 100 g were mixed with 100 mL ethanol (absolute). The mixtures were kept individually in the rotary shaker (60 rpm) for 2 days. Separate mixtures were consequently filtered using muslin cloth followed by desiccation at 55 °C. The desiccated extracts were stored at 4 °C in air tight vials. Thereafter, 100 mg of the aqueous and ethanolic extracts were dissolved in 10 mL sterile distilled water and 10 mL of dimethyl formamide (DMFO, Sigma-Aldrich) respectively and filtered through 0.45 μm filter for the examination of microbial assay.

2.2. Bacterial and fungal cultures

The MRSA and MDR isolates of Escherichia coli, Streptococcus pyogenes, Candida auris and Trichophyton rubrum were obtained from our clinical isolate collection at Department of Microbiology, College of Applied Medical Sciences, King Khalid University, Abha, Saudi Arabia. The isolates were previously identified using recommended morphological and biochemical tests (data not shown). The bacterial cultures were maintained in Muller Hinton broth media (MHB) at 37 °C and preserved in nutrient agar slopes at 4 °C till use. The fungal cultures were maintained at 28 °C in Sabouraud Dextrose Agar with Chloramphenicol 0.05% (SDA) and subsequently sub-cultured in potato dextrose agar (PDA) for inducing sporulation. Antibiotic sensitivity pattern was determined by Bauer et al. method (Bauer et al., 1966).

2.3. Disc diffusion method

The MRSA and MDR bacterial isolates were surface swabbed in Muller-Hinton agar (MHA) plates with 100 μL of the logarithmic phase bacteria and fungi at a concentration set to 0.5 McFarland turbidity standard (10⁸ cfu/mL). Previously prepared aqueous and ethanolic Coccinia extract saturated 0.7 cm discs were placed onto the plates with 2 cm space and the plates were kept at 37/30 °C for 28/48 h for bacterial and fungal antimicrobial potency, respectively. MIC was inferred as the least concentration of the extracts that prevented the visible growth “zone of clearance” of the bacterial cultures (Harish et al., 2010). For the sensitivity test of Candida, MHA with 2% glucose and 0.5 μg/mL methylene blue dye (MHA-GMB) was used. Where the yeast suspension was prepared using sterile 0.85% saline and the turbidity adjusted to 0.5 McFarland standards with concentration 1 × 10⁶ cells per mL. A semiconfluent lawn culture was made using sterile cotton swab and the extract saturated discs were placed.

2.4. Tube dilution method

Traditional tube dilution method was followed for antibacterial and antifungal screening. Serial dilutions of each extract from 10 mg/mL to 1 mg/mL were prepared in 2.6 mL MHB reaching a final volume of 3.6 mL. Initial OD was measured at 590 nm, to which 0.45 mL of the bacterial suspension (1×10⁶ cfu/mL) was added to make the final volume as 4 mL. Post incubation of tubes at 37 °C (bacterial) for 24–48 h and 30 °C (fungal) for 48–96 h, then the final OD was measured. The difference between the initial OD and final OD determined the inhibition potency of bacterial growth. Blank tubes containing sterile media and culture tubes containing 0.4 mL (1 × 10⁶ cfu/mL) of the bacterial or fungal suspension served as negative and positive control respectively. The MIC was calculated as the extract concentration showing fall in the OD as compared to positive control (Helal et al., 2019; Manavathu et al., 1996).

2.5. Statistical analysis

All the experiments were done in triplicate unless specified. Statistics were done with Graph Pad Prism v.6.0. Statistical significance was assessed through one way ANOVA and significances were denoted by p < 0.05. All data were presented as mean values ± standard error (SE).

3. Results

Results depicted in Tables 1 & 2 confirmed the multi-drug resistance as well as the antibiotic/fungal sensitivity pattern of the clinical isolates. From the current investigation it is evident that ethanolic extract of Coccinia indica exhibited antibacterial and antifungal bioactivity in significant manner than the aqueous extract (Table 3). Since ethanol was used for extraction, the residual traces of ethanol in the dried extracts were tested with routine alcohol identification test that gave negative results.

The results of the disc diffusion method showed that Coccinia ethanolic extract exhibited comparable activity against MRSA at 250 μg, S. pyogenes at 200 μg and C. auris at 250 μg with its antibiotic and antifungal controls (Fig. 1 & Tables 4, 5).

Indeed, concentration dependent activity against MRSA, S. pyogenes and C. auris were evident (Fig. 1 & Table 4, 5) showing a direct relationship with the active ingredients of the extract. Additionally, it may be further noted that the ethanolic extract exhibited a mild activity against E.coli (Fig. 1 & Table 4, 5) however it failed to exhibit fullest activity comparable to antibiotics used as positive con-
trol even at higher concentration (Data not shown). The Coccinia aqueous extract exhibited very low bioactivity against S. pyogenes (Fig. 2 & Table 4, 5). Concomitantly, the results of the tube dilution method augmented the results of disc diffusion method well (Figs. 3, 4 & Table 6).

To add, the reduction of OD was not observed in the aqueous extract while the statistically significant OD reduction was observed at 100 \( \mu g \) for MRSA and S. pyogenes while E. coli and T. rubrum did not show any observable activity with both aqueous and ethanolic Coccinia extracts (Figs. 3, 4 & Table 6). Further clear dose dependent reduction of OD was observed with this method is validating the result of disc diffusion too. To add, we did not test C. auris by tube dilution and T. rubrum by disc diffusion as their growth pattern was slow inversely proportional to inoculum.

To summarize (Table 3) from both disc and tube dilution method the ethanolic extract of Coccinia exhibited profound activity against Gram +ve organisms and yeast like organisms while showed little activity against Gram –ve organisms and dermatophytes or filamentous fungi.

4. Discussion

The antibacterial and fungal effects of the ethanolic extracts of Coccinia leaf extracts showed promising activity against three out of five organisms tested. MRSA, S. pyogenes and C. auris were susceptible to the ethanolic extract while aqueous extract did not have any observable activity on any of the tested organisms. E. coli and T. rubrum on the other hand were not susceptible to the both the extracts of Coccinia leaves. We used only crude extracts in this study as many of the previous literature and our

| Antibiotics | Clinical Isolates | MRSA | S. pyogenes | E. coli |
|-------------|------------------|------|-------------|--------|
| Ampicillin (A) | R | R | R |
| Amoxiclav (Ac) | R | R | R |
| Amikacin (Ak) | S | NA | R |
| Ceftazidine (Ca) | R | NA | R |
| Cefotaxime (Ce) | R | S | R |
| Ciprofloxacin (Cf) | R | NA | R |
| Cefuroxime (Cu) | R | S | R |
| Cefazolin (Cz) | R | S | R |
| Gentamicin (G) | S | NA | R |
| Imipenem (I) | NA | NA | I |
| Nalidoxic acid (Na) | NA | NA | R |
| Nitrofurantoin (Ni) | S | NA | S |
| Norfloxacin (Nx) | R | NA | R |
| Erythromycin(E) | | R | S | NA |
| Clindamycin (Cd) | | S | S | NA |
| Penicillin (P) | R | S | NA |
| Rifampicin (R) | | R | NA | NA |
| Vancomycin (Va) | | E test | S | NA | NA |

Where R: resistant; S: susceptible and NA: not applicable.

| Antifungal drugs | Clinical Isolates | C. auris | T. rubrum |
|------------------|------------------|----------|----------|
| Fluconazole | R | S | |
| Voriconazole | R | S | |
| Anidulafungin | S | S | |
| Amphotericin B | S | S | |

Where (R) stands for resistant, (S) stands for susceptible and (NA) stands for not applicable.

Table 3
Summary of antibacterial and fungal activity by both disc diffusion and tube dilution technique.

| Extracts | Concentration of the extracts in \( \mu g/mL \) | Clinical Isolates |
|----------|---------------------------------------------|------------------|
|          |                                             | MRSA | S. pyogenes | E. coli | C. auris | T. rubrum |
|          |                                             | Disc | Tube | Disc | Tube | Disc | Tube | Disc | Tube | Disc | Tube |
| Aqueous C. indica | 25 | NDA | NDA | NDA | NDA | NDA | NDA | NDA | NDA | NDA | NDA |
|          | 50 | NDA | NDA | NDA | NDA | NDA | NDA | NDA | NDA | NDA | NDA |
|          | 100 | NDA | NDA | I | I | NDA | NDA | NDA | NDA | NDA | I |
|          | 200 | NDA | NDA | I | I | NDA | NDA | NDA | NDA | NDA | I |
|          | 250 | NDA | NDA | I | I | NDA | NDA | NDA | NDA | NDA | I |
|          | 300 | NDA | NDA | I | I | NDA | NDA | NDA | NDA | NDA | I |
| Ethanol C. indica | 25 | I | I | I | I | NDA | NDA | I | ND | I | NDA |
|          | 50 | I | I | M | M | NDA | NDA | I | ND | I | NDA |
|          | 100 | M | M | S | S | I | I | ND | ND | I | |
|          | 200 | S | S | HS | HS | I | I | M | ND | I | ND |
|          | 250 | HS | HS | HS | HS | M | M | HS | ND | I | ND |
|          | 300 | HS | HS | HS | HS | M | M | HS | ND | I | ND |

Summary of antibacterial and fungal activity by both disc diffusion and tube dilution technique. Where ND: not done; NDA: no detectable activity; I: intermediate (Very marginal reduction of OD values); M: mild (Considerable reduction in OD values due to slow growth/ altered growth pattern); S: sensitive (Above 70% reduction in the OD values) and HS: highly sensitive (No detectable growth of organisms).
observation has always turned to be in favor of crude over isolated compounds as later usually does not exhibit an observable property compared to the crude extract.

It is evident from current study and other studies elsewhere (Oboh et al., 2008; Yi et al., 2012) that, the ethanolic extracts usually have higher efficacy and activity compared to the aqueous extracts for the fact that, the former contains more secondary metabolites (Yi et al., 2012) extractable in ethanol and other higher polar solvents (Abubakar and Haque, 2020) than the aqueous extraction. Traditionally aqueous extracts of leaves served mostly as topical applications for bacterial infections (Cowan, 1999) and in advanced cases alcohol was used for extraction. The results of the ethanolic extract clearly indicated the presence of soluble metabolites resulting in dose dependent activity (Gonelimali et al., 2018). These observations well corroborated with the literature evidences preferring ethanolic extracts over aqueous extracts used at higher concentrations (Chassagne et al., 2020) and for longer periods (Silva et al., 2016) in treating infectious diseases.

Table 4
Antibacterial and fungal activity by disc diffusion method.

| Herb          | Nature of extract | Clinical Isolates | Concentration (µg/mL) of the extract versus Zone of Clearance (mm)* |
|---------------|-------------------|-------------------|------------------------------------------------------------------|
|               |                   |                   | 25     50    75       100       200       250       300       |
| Coccinia indica| Aqueous           | MRSA              | +/-    +/-    +/-       +/-       +/-       +/-       +/-       |
|               |                   | S.pyogenes        | +/-    +/-    +/-       4         4          4          4          |
|               |                   | E.coli            | +/-    +/-    +/-       +/-       +/-       +/-       +/-       |
|               |                   | C.auris           | +/-    +/-    +/-       +/-       +/-       +/-       +/-       |
|               | Ethanolic         | MRSA              | 7       8       10       14        18        19        19        |
|               |                   | S.pyogenes        | 4       5       10       13        19        19        19        |
|               |                   | E.coli            | +/-    +/-    +/-       5         7          11        12        |
|               |                   | C.auris           | 2       5       9        10        14        18        18        |

*The results are expressed as average zone of inhibition (mm) from three independent experiments. Where (-/-) stands for Nil clearance.

Table 5
Reference sensitivity of positive controls used along with the disc diffusion test.

| Antibiotics | Zone of Clearance (mm)* |
|-------------|-------------------------|
|             | MRSA  | S.pyogenes | E.coli | C.auris |
| Gentamicin (G) | 19    | NA        | NA     | NA      |
| Erythromycin (E) | NA    | 19        | NA     | NA      |
| Nitrofurantoin (Nf) | NA    | NA        | 18     | NA      |
| Anidulafungin (Ani) | NA    | NA        | NA     | 18      |

Where NA: not applicable.

Fig. 2. Antibacterial and fungal activity of aqueous extract of Coccinia indica by disc diffusion method. Zone of clearance is measured in mm and compared to the susceptible control antibiotics and antifungal drug.

Fig. 3. Antibacterial and fungal activity of aqueous extract of Coccinia indica by tube dilution method. Reduction in the optical density (OD) of the extract added tubes are compared to the positive control (tube with only organism).

Fig. 4. Antibacterial and fungal activity of aqueous extract of Coccinia indica by tube dilution method. Reduction in the optical density (OD) of the extract added tubes are compared to the positive control (tube with only organism).
instance, the ethanolic extract showed profound activity over gram +ve and yeast like organisms than gram -ve or filamentous fungi. Further this property may be attributed to the secondary metabolites exhibiting specific mechanism over gram +ve organisms (Gorlenko et al., 2020; Jakubiec-Krzesniak et al., 2018). These results were little varied with classical understanding of the antibacterial or antifungal studies (Bhalodia and Shukla, 2011). It may be noted that crude extracts usually posses’ mixture of chemical constituents which usually exhibits mixed activity when tested on broad range of microorganisms inclusive bacteria, viruses, fungi and parasites (Noorulla et al., 2009; Sakharak and Shukla, 2011). Though selective and specific activities over organisms are not common phenomenon observed with herbal extracts, it is evidenced form the observation that both aqueous and ethanolic extracts of Coccinia showed a feeble activity over E.-coli. However, the results could be escalated to complete inhibition of E.coli. This may be due to less availability of the metabolite in the ethanol extract or loss of activity due to ethanol has to be further investigated with the use of higher polar solvents for the extraction.

It may be noted that some of important bioactivity of Coccinia fruits has been documented (Kondhare and Lade, 2017; Shaheen et al., 2011) over metabolic diseases and some studies have been undertaken in Coccinia indica and other subspecies over various microorganisms. However, emphasis over antibiotic resistant organisms of both bacteria and fungi have not been addressed with herbal extracts. Even with few of such studies, the results have not been escalated to higher trials nor authenticated to be used in the therapy. The ethanolic extract of Coccinia turned out be a good candidate to be further tested for antibiotic resistant gram +ve bacteria and yeast like fungi to deduce the mechanism of the action.

5. Conclusion

The results of the ethanolic extracts of Coccinia indica over all the five organisms by both disc diffusion and tube dilution methods were comparative and results were well in agreement with each other. The extracts were move active over gram +ve and yeast like organisms compared to gram –ve and filamentous fungi. The potential application of ethanolic extracts specially to treat gram +ve organisms and candida will be an addition to the list of lead molecules from the natural origin to combat drug resistance in common. To add, future analysis would be warranted to authenticate the bioactivity of this leaf extract in higher disease models.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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