Cytotoxic and antimicrobial potential of different leaves extracts of *R. fruticosus* used traditionally to treat diabetes

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ABSTRACT  
Medicinal plant as herbal medicine is widely used to cure infectious diseases. *Rubus fruticosus L. (R. fruticosus)* has been studied for its antimicrobial and cytotoxic activities. Different polarity leave extracts were prepared by using hexane, chloroform, ethyl acetate and hydro alcoholic solvents. Agar diffusion method has been used to assess the antibacterial activity against two gram-positive *Enterococcus faecalis* and *Staphylococcus aureus* and two gram-negative *Escherichia coli* and *Haemophilus influenza* bacterial strains. Cytotoxic activity was carried out against brine shrimp using the nauplii method (BSL). The results so obtained for various experiments demonstrates the total number of extracts produces moderate to strong antibacterial activity against the gram (+ and -). The best activity was discovered within hydro alcoholic upon all concentrations, whereas the inhibition zone exists within the range of 6 – 11 mm. In addition to that, the cytotoxic activity test confirms that hydro alcoholic extracts the maximum toxicity for values LC50 (4.68–6.96 μg/ml), where the LC50 values for all extracts be located within the range of 4.68-6.96 μg/ml. Finally, the plant itself and its derived extracts have been used as a folk medicine to treat serious infectious diseases.

1. Introduction

For hundreds of thousands of years, humans have been using the natural resources for different purposes, including medicinal and nutritional. Indigenous communities living in biodiversity-rich areas have acquired very valuable knowledge of medical plant utilization and conservation. This kind of traditional knowledge has been enhanced over the years through observation and experimentation. A certain plant or plants in general has a huge pharmacological and therapeutic potential as it can fulfill the requirement with minimum side effects compared to synthetic drugs [1,2]. Ingredients are vital molecules of plant and its products which seem to be major alternatives to control the pharmacological and biological activity against the pathogenic Gram (+ and −) bacterial strains [3]. Generally, the plant ingredients played a vital role in the discovery of drugs to treat serious infectious diseases. The pharmacological and biological importance of that particular plant depends on the ingredient which can make a specific pharmacological effect on the human body. Those biologically active ingredients are chalcone, alkaloids, flavonoids, tannins and phenolic compounds. Furthermore, the plant constituents would be biologically helpful to discover the folkloric remedies with significant activity. Usually, the phytochemical of higher plants has been considered as an acceptable and efficient approach to the study of new serious infectious disease and cancer drugs [4].

*R. fruticosus L. (Rosaceae)* is a medicinal plant which is locally called ’fursad’ [5], is a seasonal well-known tree owing to its famous fruits. Sometimes, the plant gives red, violet, white or black fruits, which is called blackberry due to its black colour. It is an important traded crop globally because of the delightful taste, enjoyable flavor and dietary profile. It is theorized that the country of origin of this plant is Armenia and it is widely grown in European countries, northern areas of Pakistan, Saudi Arabia, and Oman [6–9]. Oman is considered as one of the most advanced countries regarding ethno-botanical knowledge due to the presence of multiethnic groups of ancient lineage and the occurrence of very diverse vegetation. Furthermore, Oman is among the world’s leading bio diversified areas due to the presence of different plant species where some wild plant species have been utilized by Omani communities to meet their different requirements recorded so far. The selected plant belongs to a large family *Rosaceae*. The genus *Rubus* comprises about 700 species with 12 subgenera [10,11]. *R.
fruticosus is a semi-prostrate to almost erect, grows up to 2 m. It is a bushy plant with thorns, but some of the cultivated species have different types of thorns [12]. The survival of blackberries is about three to five seasons [14]. The arrangement of the leaves is tri or penta foliate (Fig. 1). The flowers are white in colour, in a dense short branched clusters and fruits are black [15]. The plant is used for different purposes, such as herbal medicine, cosmetic and nutritive [13]. It is used in pharmacology as an antioxidant and as an anti-carcinogenic [16] agent. Several studies have been tackled to show that, the leaves of the Rubus tree balance blood and sugar and help to treat type-2 diabetes by stimulating the pancreas for insulin secretion. Eating fresh Rubus leaves gives the body a sense of satiety, so it is a good choice for those who seek to lose their excess weight without the risk of any complications. However, the use of the fruit in cases of harsh diet or its overuse should be avoided. They also help to improve immunity [17], the fruit works as anti-inflammatory, anti-diabetic, and antiviral [18], antimicrobial [19] in a human organism. Fursad is also used as a stimulating agent during the quarantine. In addition, the herb is used to treat diarrhea [20]. In Europe, since old times, R. fruticosus has been used to cure diabetes. The leaves extract demonstrated a significant hypoglycemic impact on diabetic rats [21]. The aqueous fruit extract of the selected plant showed hyaluronidase inhibitory activity [22]. Nowadays, its fruits are used to make delicious foods, such as jam, wine, tea, ice-cream, deserts, seedless jellies and bakery products [23]. R. fruticosus contains various phytochemicals, such as alkaloids, flavonoids, tannins, sapo-nins, glycosides, terpenoids, steroids, and carbohydrates [24,25]. The plant also contains ascorbic acid, organic acids, tannins, and volatile oils [26]. It also contains numerous phytochemicals including poly-phenols, anthocyanins, salicylic acid, ellagic acid, and fiber [27,28]. Several studies related to R. fruticosus in have been conducted, while only a few studies reported for the selected species in Oman. The main challenge is the lack of prior research studies into this plant, which is an important reason of choosing this study. Kanegusuku et al. proved that methanol, hexane and ethyl acetate extract showed significant cyto-toxic activity against brine shrimp [29]. Bhagat and Thusoo stated that the fruit methanol extract of the selected plant was more active against lung cancer cells (A549) than the extract of the leaves part with 72 % growth inhibition at 100 μg/ml concentration [29]. Riaz et al. stated that the possible source of antibacterial activity of methanol extracts from various parts of the plant against eight bacterial strains. Their results showed that all extracts gave significant growth of inhibition. Their results also showed that potency on minimum inhibitory concentration was stem > root > leaves > fruit extract [5]. Another study on the juice of blackberry inhibits the growth of Bacillus cereus, Bacillus subtilis, Streptococcus marcescens and Escherichia coli with percentages varying from 50 to 75 % [30]. Therefore, the goal of this study is to prepare different leaves extracts and assess their antimicrobial and cytotoxic behaviour using agar diffusion and brine shrimp nauplii methods.

2. Materials and methods

2.1. Materials

Petroleum ether, ethanol (100 %), methanol, hexane, chloroform, and ethyl acetate, dimethyl sulfoxide were obtained from Sigma Chemical Company, Germany. The shrimp eggs (ARTEMİCYS'TS) were bought from GOAOVA, USA. The sea salt was collected from a local market. Whatmann filter paper was used as a disc (5 mm diameter). The agar and plastic Petri dishes were bought from ScharlauChemie Company, India. Silica gel and pre-coated TLC were collected from E. Merck, Germany.

2.2. Bacterial strains

Both Gram + and Gram- bacteria were used in the present experiment. Total four bacteria, two gram-positive Enterococcus faecalis (E. faecalis) and Staphylococcus aureus (S. aureus) and two gram-negative Escherichia coli (E. coli), Haemophilus influenza (H. influenza) which were collected from Nizwa Hospital and cultured in the Microbiology Department, College of Arts and Sciences, University of Nizwa.

2.3. Collection and identification of plant samples

In November 2016, the leaves of R. fruticosus have been collected from Barka, Sultanate of Oman. The samples were moved to the laboratory and well cleaned and dried in the shade. The plant sample was identified by Dr. Syed Abdullah Gilani, Taxonomist, College of Arts and Sciences, at the University of Nizwa.

2.4. Preparation of crude extracts

The dried leaves were powdered (465 g) and macerated in methanol (1200 ml) for one week. The methanol was evaporated at 40°C by using a rotatory evaporator to a concentration. Both Gram + and Gram- bacteria were used in the present experiment. Total four bacteria, two gram-positive Enterococcus faecalis (E. faecalis) and Staphylococcus aureus (S. aureus) and two gram-negative Escherichia coli (E. coli), Haemophilus influenza (H. influenza) which were collected from Nizwa Hospital and cultured in the Microbiology Department, College of Arts and Sciences, University of Nizwa.

2.5. Cytotoxic activity of leaves extracts

In vitro cytotoxicity assay was done on shrimps of Artemiasalina as described by Said et al. and Olaru et al. [31,32].

2.5.1. Hatching of shrimp larvae

The eggs (Artemiasalina) (50 mg) were hatched in sea-water (3.8 % solution of NaCl) at room temperature in a tank made of plastic separated into two compartments with a perforated polyethylene wall, one of the compartments was covered, to maintain artificial darkness. In addition, the other compartment is illuminated to attract the larvae of the shrimp to transfer from the dark compartment 24 h later, the larva’s (nauplii) was collected [30].

2.5.2. Brine shrimp lethality assay

Solutions of the extracts were prepared in 5 % dimethyl sulphoxide (DMSO), at various concentrations 10, 8, 5 and 3 μg/ml. One milliliter from each concentration was incubated in triplicate vials with the shrimp larvae in a total volume of 5 ml. Ten shrimp larvae were placed in each of the triplicate vials. Others were placed in a mixture of DMSO (5 %) and seawater, to serve as a negative control. The same concentrations of potassium permanganate (10, 8, 5 and 3 μg/ml) were used as a positive control. After 24 h incubation, the survival nauplii were inspected against a lighted background. The mortality (%) was plotted against the various concentrations. The concentration of killing fifty percent of the larvae (LC50) was determined from the graph [31].
2.6. Antibacterial activity

A stock solution was prepared for every single extract and exposed to the dilution technique, by dimethyl sulfoxide (DMSO) as a solvent to provide 2000, 1000, 500, and 250 μg/ml concentrations. Ciprofloxacin 250 μg/ml as standard was all set by using a similar solvent which was used for dilution. Twenty agar plates were prepared by the distribution of each type of bacteria over four plates. After that, filter paper discs (5 mm) were dipped in each concentration and then placed on the agar plates inoculated with the bacteria. Finally, the plates were placed in the incubator for 24 h at 37 °C. Antibacterial activity was assisted by measuring the diameter of the inhibition zone against the tested bacteria.

2.7. Fractionation of hexane extract by column chromatography (CC)

The hexane extract was loaded on a silica column. The gradient elution was carried out using hexane, hexane/chloroform, chloroform/ethyl acetate and ethyl acetate/methanol solvent mixtures. The collected fractions were analysed by TLC. Those of a similar manner were combined together and checked for the cytotoxic activity to determine the best fraction. Similarly, the hydroalcoholic extract that has the highest potential of bacterial inhibition was chromatographed over a column (3.5 × 75 cm, silica gel 200 g). The elution was started with petroleum ether and the polarity was increased gradually with chloroform, ethyl acetate, and methanol. The collected fractions were checked by TLC using petroleum ether: ethyl acetate (40 %) mobile phase, and similar fractions were combined to give four main fractions. All these fractions were checked for antibacterial activity against the two gram-negative and two gram-positive bacteria.

3. Results and statistical analysis

The leaves powder of R. fruticosus samples were extracted by using an extractive Maceration technique which is conducted at room temperature. It involves of submerging a leave powder of R. fruticosus in a liquid of a methyl alcohol for seven days, for a variable time based on the plant material (leaves powder); methanolic residue weighed about 34.42 g was fractionated of which gave 8.1 g represents nonpolar compound and 21.2 are polar compounds, since they came with the two polar solvents.

Thus, the yields of semi-solid residues after extraction and evaporation from 465 g of the dried powder leaves of R. fruticosus have been obtained using the standard formula,

\[
\text{Yield} \% = \frac{\text{extracted weight } \times 100}{\text{leaf powder weight}}
\]

Table 1, displays the yields percentage for the extractions, that expressed as the value of mean plus-minus gm.

3.1. Brine shrimp lethality test

In general, the Brine shrimp lethality test is simple and widely used in the evaluation of cytotoxicity of medicines especially natural plant extracts, based on the killing ability of compounds on a simple organism—brine shrimp (Artemiasalina) [32].

The cytotoxic activity was assessed for each extract at different concentrations by a brine shrimp method with modification [29,30]. The parent methanol extract and the derived fractions of hexane, chloroform, ethyl acetate, and hydroalcoholic have shown potent behavior against the brine shrimp larvae. The mean percentage mortality of brine shrimp larvae for various extracts of R. fruticosus has been shown in Table 2. The activity order was hydro alcoholic > hexane = chloroform > ethyl acetate > methanol, with concentrations that killed 50% (LC50) ranging from 4.68 to 6.96 (μg/ml) [32–35], which is even lower than that of the positive control potassium permanganate and negative control mixture of DMSO (5 %) and seawater in Table 3.

The six fractions obtained from the column chromatography of the hexane extract were collected Fr.1 (0.26 g), Fr. 2 (1.01 g), Fr. 3 (2.12 g), Fr. 4 (0.42 g), Fr. 5 (0.71 g) and Fr. 6 (0.45 g). All fractions were subjected to brine shrimp test and the results in Table 4 indicates that all fractions contain compounds that are cytotoxic with LC50 between 49.76–69.01(μg/ml) which is higher than that shown by the crude extracts.

3.2. Antibacterial activity

The disc diffusion method is a test of the antibiotic sensitivity of bacteria, which is used to evaluate the antibacterial properties of each extract and to determine the best antibiotic against a new or drug-resistant. In this method, the concentration gradient of each extract in the nutrient medium was prepared and grew. The growth of the bacteria has been observed for seeded bacteria in the agar plates. The amount of space around every filter paper disc indicates the lethality of that concentration of that extract on the bacteria in question. Table 5, shows the clear zone of inhibition (in mm) of growth bacteria considering a measure of antibacterial activity of different extracts of leaves of R. fruticosus against E. coli, H. influenza, E. faecalis, and S. aureus for different concentrations 250, 500, 1000, and 2000 (μg/ml).

4. Discussion

In this article, we have successfully incorporated the concepts of Cytotoxic and antimicrobial potential of different leaves extracts of R. fruticosus used traditionally to treat diabetes. The methanol extract and its derived fractions were deployed to determine their toxic activity against the brine shrimp larvae. The experimental results show that the high concentration of each extract gave a promising activity against the brine shrimp larvae. Moreover, the highest activity was recorded during the use of hydro alcoholic extract. The use of all extracts at different concentrations are presented in Table 2. In addition, the range of LC50 values among the extract in between is about 4.68–6.69(μg/ml). According to the toxicity, the hydro alcoholic extract illustrates the highest activity and the lowest is the parent methanol extract. That means, the hydro alcoholic extract contains the maximum numbers, as well as the highest concentration, of cytotoxic compounds. Hexane extract showed very high toxicity and all fractions of hexane extracts also showed promising activity against brine shrimp. Among them, fractions 3 and 6 gave significant toxicity compared to the other fractions. That means, most of the toxic ingredients could be found in those two fractions (Table 4). Phytochemicals analysis showed that the selected plant species contains alkaloids, flavonoids, tannins, saponins, glycosides, terpenoids, sterols, and carbohydrates [24,25]. The plant also contains ascorbic acid, organic acids, tannins, and volatile oils [26]. It also contains numerous phytochemicals including polyphenols, anthocyanins, salicylic acid, ellagic acid, and fiber [27,28]. Therefore, we can easily isolate and identify the cytotoxic ingredients from those fractions. The results so obtained have been different from those reported by the other authors depends on several issues, such as the natural environment in Oman being different from the natural environment.
environment in the reported countries; the procedures of extraction from the leaves are not the same, and, due to the heat, as well as the type of the extraction process, the chemical ingredient could change or breakdown into toxic ingredients [36,37]. While for antimicrobial activity, Table 5, shows all extracts, methanol, hexane, chloroform, ethyl acetate and hydro alcoholic extracts, determined in their antimicrobial activity by using an agar gel method against two gram-negative E. coli, and H. influenza and two gram-positive E. faecalis and S. aureus bacteria. The average range of activity of all extracts against the applied bacterial strains is within the range of 6–11 mm. Though, it is stimulating that all the extracts at all concentrations gave significant activities against the gram-positive bacteria E. faecalis. But the other gram-positive bacteria S. aureus did not give any activity against the applied bacteria and E. coli, and H. influenza and two gram-positive E. faecalis and S. aureus bacteria. The average range of activity of all extracts against the applied bacterial strains is within the range of 6–11 mm. Though, it is stimulating that all the extracts at all concentrations gave significant activities against the above-mentioned bacteria. Fraction 1 did not give any activity. Fraction 2 is active against the S. aureus but the other bacteria are not responding. Fraction 3 gave aproming activity against both gram-positive bacterial strains within the range of 0–20 mm. The highest activity which is 20 mm, was obtained from Fraction 3 at concentration 2000 μg/ml. While, in Table 6 shows that, A fraction 4 gave a very good activity against all gram (+ and -) bacterial strains.

5. Conclusion

In this study, the cytotoxic and antibacterial activity of the Rubus fruticosus has been investigated. All extracts showed excellent cytotoxic activity against brine shrimp larvae indicating that the presence of compounds is bioactive against the brine shrimps. Remarkably, the fractions of the hexane extract were of higher LC50 (LC50 49.76–69.01 μg/ml) than those shown by the hexane extract (LC50 5.89 μg/ml). For the antibacterial activity, extracts of methanol, hexane, chloroform, ethyl acetate and hydro alcoholic are effective against

Table 2
Mean percent mortality of brine shrimp larvae when exposed to methanol, hexane, chloroform, ethyl acetate and hydroalcoholic extracts from R. fruticosus. (n = 10 larvae per treatment).

| Extract Conc.(μg/ml) | Mean percentage mortality of brine shrimp larvae % |
|----------------------|-----------------------------------------------|
|                      | Standard KMnO4 | Methanol | Hexane | Chloroform | Ethyl acetate | Hydroalcoholic | DMSO (5 %) |
| 10                   | 40             | 90       | 100    | 90         | 100           | 70             | 0         |
| 8                    | 20             | 50       | 80     | 60         | 80            | 50             | 0         |
| 5                    | 10             | 30       | 30     | 40         | 40            | 50             | 0         |
| 3                    | 0              | 10       | 20     | 30         | 60            | 60             | 0         |

n = 10 larvae per treatment.

Table 3
LC50 of methanol, hexane, chloroform, ethyl acetate and hydroalcoholic extracts from R. fruticosus against brine shrimp larvae (n = 10).

| Extract             | LC50 (μg/ml) |
|---------------------|--------------|
| KMnO4               | 12.58 ± 0.43 |
| Methanol            | 6.96 ± 0.98  |
| Hexane              | 5.88 ± 0.13  |
| Chloroform          | 5.89 ± 0.31  |
| Ethyl acetate       | 6.15 ± 0.46  |
| Hydroalcoholic      | 4.68 ± 0.19  |
| DMSO + H2O          | 0            |

n = 10 larvae.

Table 4
LC50 of different fractions from column chromatography of hexane extract of R. fruticosus leaves against brine shrimp larvae (n = 10).

| Fraction nos | LC50 (μg/ml) |
|--------------|--------------|
| 1            | 64.67 ± 0.17 |
| 2            | 64.67 ± 0.42 |
| 3            | 49.76 ± 0.69 |
| 4            | 64.67 ± 0.25 |
| 5            | 69.01 ± 0.88 |
| 6            | 54.44 ± 0.09 |
| KMnO4        | 19.59 ± 0.18 |

n = 10 larvae shrimp.

Table 5
Antimicrobial activity of different extracts of aerial parts of R. fruticosus against E. coli, H. influenza, E. faecalis and S. aureus (diameter of inhibition zones in mm).

| Extracts | Conc. (μg/ml) | E. coli | H. influenza | E. faecalis | S. aureus |
|----------|---------------|---------|--------------|-------------|-----------|
| Methanol | 250           | 6 ± 0.10| 8 ± 0.09     | 6 ± 0.17    | nd        |
| 500      | 6 ± 0.29     | 6 ± 0.13| 10 ± 0.33    | 6 ± 0.27    | nd        |
| 1000     | nd           | 7 ± 0.45| 7 ± 0.15     | 6 ± 0.11    | nd        |
| 2000     | 10 ± 0.24    | 10 ± 0.71| 10 ± 0.11   | 6 ± 0.72    | nd        |
| Ciprofloxacin | 250     | 30 ± 0.45| 23 ± 0.23    | 23 ± 0.51   | 23 ± 0.12 |
| Hexane   | 250           | 9 ± 0.09| 6 ± 0.16     | 7 ± 0.22    | nd        |
| 500      | 7 ± 0.77     | nd       | 7 ± 0.89     | 6 ± 0.14    | nd        |
| 1000     | 10 ± 0.50    | nd       | 7 ± 0.14     | 6 ± 0.11    | nd        |
| 2000     | 9 ± 0.31     | 6 ± 0.19 | 9 ± 0.27     | 6 ± 0.72    | nd        |
| Ciprofloxacin | 250     | 31 ± 0.69| 22 ± 0.21    | 20 ± 0.25   | 21 ± 0.81 |
| Chloroform | 250          | 6 ± 0.12| nd           | 6 ± 0.14    | nd        |
| 500      | 9 ± 0.07     | nd       | 10 ± 0.33    | 6 ± 0.27    | nd        |
| 1000     | nd           | 6 ± 0.53| 7 ± 0.77     | 6 ± 0.17    | nd        |
| 2000     | 14 ± 0.11    | 6 ± 0.18| 10 ± 0.09    | 6 ± 0.17    | 6 ± 0.72 |
| Ciprofloxacin | 250     | 27 ± 0.15| 26 ± 0.18    | 23 ± 0.21   | 20 ± 0.42 |
| Ethyl acetate | 250        | nd      | 7 ± 0.13     | 7 ± 0.17    | nd        |
| 500      | 9 ± 0.32     | 6 ± 0.19| 10 ± 0.08    | 7 ± 0.77    | nd        |
| 1000     | 15 ± 0.14    | 7 ± 0.17| 9 ± 0.44     | 7 ± 0.17    | nd        |
| 2000     | 21 ± 0.71    | 8 ± 0.45| 9 ± 0.32     | 7 ± 0.17    | nd        |
| Ciprofloxacin | 250     | 29 ± 0.11| 26 ± 0.10    | 25 ± 0.19   | 21 ± 0.22 |
| Hydro alcoholic | 250         | 6 ± 0.14| 10 ± 0.09    | 10 ± 0.17   | 11 ± 0.11 |
| 500      | 9 ± 0.18     | 9 ± 0.42| 9 ± 0.68     | 10 ± 0.09   | 10 ± 0.49 |
| 1000     | 6 ± 0.08     | 9 ± 0.75| 10 ± 0.10    | 10 ± 0.43   | nd        |
| 2000     | 9 ± 0.15     | 8 ± 0.18| 10 ± 0.88    | 6 ± 0.42    | nd        |
| Ciprofloxacin | 250     | 29 ± 0.28| 30 ± 0.15    | 23 ± 0.17   | 19 ± 0.10 |
| DMSO     | 0            | 0        | 0            | 0           | nd        |

nd = Not detectable.
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.

Appendix A. Supplementary data

Supplementary material related to this article can be found in the online version, at doi:10.1016/j.toxrep.2020.01.006.

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