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Original Article:

Chemical Composition, Antioxidant Potential, and Antimicrobial Activity of *Nannorrhops ritchiana* (Griff) Aitch

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Abstract

Introduction: *Nannorrhops ritchiana* (Griff) Aitch (Arecaceae) is an edible, extensively used shrub to small tree dispersed in Iran, Afghanistan, Pakistan, and Arabian Peninsula. In this study, the total phenolic and flavonoid contents, antioxidant capacity, and antimicrobial activity of the fruit and seed extracts of *Nannorrhops ritchiana* were evaluated *in vitro*.

Materials and Methods: The plant sample was collected from a wild population in Saraydan village, Iranshahr, Sistan and Baluchistan, and the methanolic extracts of the seeds and fruits were obtained by the maceration method The Folin-Ciocalteu and aluminum chloride colorimetric protocols were followed for quantifying total phenolic and flavonoid contents respectively. The total antioxidant and antimicrobial activity of alcoholic extracts were also measured using 2,2-diphenyl-1-picrylhydrazy (DPPH) free radical scavenging assay and disc diffusion method, respectively. Antimicrobial activity was investigated on 5 Gram-positive (*Staphylococcus aureus Streptococcus, pneumoniae, Rhodococcus equi, Bacillus subtilis subsp. Spizizenii* and *Enterococcus faeca*) and 3 Gram-negative (*Shigella flexneri, Proteus vulgaris,* and *Salmonella typhi*) bacteria, and 3 fungi (*Fusarium oxysporum, Aspergillus fumigatus,* and *Candida albicans*).

Results: The total phenolic content of the seeds was higher than that of fruit (331.33±8.3 vs 360.93±10.23 µg gallic acid equivalents/g, respectively) while, the total flavonoid content of seeds was almost half as much as that of fruits (70.50±3.1 vs 127.70 ±5.25 µg quercetin equivalents/g, respectively). The ethanolic seed extract exhibited better scavenging ability for DPPH with IC_{50} of 117.77 ± 6.3 µg/mL compared to fruit extract with IC_{50} of 204.34±7.59 µg/mL. Moreover, the ethanolic extracts at initial concentrations inhibited the growth of several tested pathogenic bacterial and fungal strains.

Conclusion: These findings suggest that the fruits and seeds of *Nannorrhops ritchiana* are rich sources of phenolic compounds with natural moderate antioxidant and antimicrobial properties which can be a candidate for possible dietary and/or medication applications.

Keywords: Anti-infective agents, Antioxidants, Flavonoids, *Nannorrhops ritchiana*, Plant extracts

1. Introduction

Nannorrhops ritchiana (Griff) Aitch. (Arecaceae) is distributed naturally in the Saharo-Sindian region including India, Pakistan, Iran, Afghanistan, and Arabian Peninsula [1], and is cultivated for ornamental purposes in other parts of the world. The genus name is derived from two Greek words nannos (small) and rhops (shrublet), indicating its life form, while the specific epithet honors the English naturalist, Joseph Ritchie (1788 – 1819). The genus was considered monotypic until 2016 when a new species from Iran was introduced as *Nannorrhops baluchestanica* Khodashenas [2]. *Nannorrhops ritchiana* is a very attractive xerophyte shrub, up to 6 m high, with flabellate and cuneate leaves, branched terminal Inflorescence, and globose or ovoid drupe [1]. *Nannorrhops ritchiana* is locally called DAAZ, wild palm, or PORK in Persian, but it is commonly known as Mazari palm in literature. The plant plays a key role in the life of local people and all parts of the plant are used widely for various purposes. It is possibly the hardiest palm [3] and can be used in building construction. The fresh leaves are both firm and flexible and are used for creating handicrafts such as baskets, brooms, slippers, bracelets, small boxes as well as firm ropes for fastening heavy clusters of dates. The fruits are edible and are consumed orally for laxative [4] and purgative [5] purposes, and the seeds are used to make prayer beads. The large apical meristem at the tip of the stem is very delicious and is called BASTOOK by local people; the young leaves are also eaten as a vegetable, raw or cooked. Indigenous plants are of prime interest to scientists as sources of nutritional and pharmacological compounds. *Nannorophs ritchiana* is used orally as a decoction or infusion for treating diabetes, dyspepsia dysuria, or topically for rheumatism in Pakistan [6-7]. A huge number of plants that are traditionally used in medicine have been screened for chemical composition and antimicrobial properties worldwide, yet many remain to be studied in the future. At present, due to the increasing emergence of multidrug-resistant pathogens, the discovery of new and natural anti-microbial compounds is highly appreciated.

Before the discovery of the first antibiotic in 1928, plant materials were the only weapon to fight against pathogenic microbes. Even though antibiotics have played a phenomenal role in treating infectious diseases over decades, it has been increasingly documented that the efficacy of antimicrobial drugs is decreasing as a result of incautious drug application in disciplines like agriculture, medicine, and veterinary [8-10]. There is a pressing need for discovering natural products with high efficacy against a wide spectrum of microbes. There is growing evidence that some plant compounds not only show effective action against resistant pathogens but also reverse the resistance of microbes to antibiotics and augment the effectiveness of classical antibiotics synergistically [8, 11-13].

Numerous studies have shown a positive correlation between the antimicrobial properties and antioxidant capacities of various organs of plants. The antioxidant capacity of molecules plays a crucial role in cell detoxification and the prevention of many pathological disorders caused by free radicals, including depression, diabetes, gastrointestinal and neurological problems, heart and respiratory tract diseases, tumors, and aging [14-16]. In plants, the antioxidant activity comes largely from the flavonoid and phenolic contents of the organs; thus, the higher the total flavonoid and phenolic compounds, the higher the antioxidant capacity of the plant will be [17,18]. The flavonoid and phenolic compounds play key roles in plant development and protection processes. Their hydroxyl groups contribute to the antioxidant action by scavenging free radicals [19].

Here, the total flavonoid and phenolic contents and antioxidant capacity of *Nannorrhops ritchiana* were assayed *in vitro*. Also, the efficacy of crude methanol extracts of seeds and fruits of the species was tested against three Gram-positive, three Gram-negative bacteria, and two fungi to evaluate their potential as nutritional and/or antimicrobial agents.

2. Materials and Methods

Plant Sample Preparation

In this descriptive cross-sectional study, the seeds and fruits of *Nannorrhops ritchiana* were gathered in October 2020 from a wild population in Saraydan village (coordinates 27°15′44″N 60°55′26″E), Iranshahr, Sistan and Baluchistan (Figure 1). The first author of the plant was confirmed by the second author and a herbarium voucher was prepared and deposited in the Herbarium of the University of Zabol, under herbarium code: 1501. Fruits and seeds were separated and air-dried in shade for a week. The fruit of *Nannorrhops ritchiana* is botanically a berry with a membranous endocarp and a fleshy mesocarp. Eventually, the seeds and fruits were powdered using a blender and were stored at 4° C in the refrigerator.

Total phenolic content assessment

The total phenolic contents of the seeds and fruits were determined by employing the Folin-Ciocalteu protocol [20] in which 0.5 g of each plant organ powder was added to 1 ml of pure methanol in separate test tubes. The test tubes were placed on a shaker overnight. Then, the tubes were placed in the dark for another 24 hours. An amount of 0.2 ml of each extract was added to 2.7 mL distilled water and 0.1 mL Folin-Ciocalteu reagent in distinct tubes. After five minutes, 5 mL of aqueous sodium carbonate solution (70 g/l) was loaded to each tube, followed by incubation for 1.5 hours at ambient temperature and pressure. This method was copied for different concentrations of gallic acid (50, 100, and 200 µg/mL) to construct a standard graph of light absorbance versus solution concentration. The absorbance of all mixtures was recorded at 760 nm *via* a 6405 UV/Vis Jenway spectrophotometer. The equation of straight lines was determined as y = mx+b. The absorbance of each extract (y) was inserted into the equation to obtain an equivalent concentration (y). The absorbance reading was repeated three times for each solution and the total phenolic content was reported as µg gallic acid equivalent (GAE) per gram of dried extract according to the following equation:

$$TPC = y \times v/ m$$

Where v and m are the volume of initial extract in ml and the weight of plant extract in g, respectively.

Total flavonoid content assessment

The total flavonoid content of fruits and seeds of *Nannorrhops ritchiana* was assessed based on the widely accepted aluminum chloride colorimetric protocol [21]. Quercetin was used for producing the calibration curve. According to the protocol, 0.5 g of each plant organ powder was added to 1 ml of pure methanol in separate test tubes. The test tubes were placed on a shaker

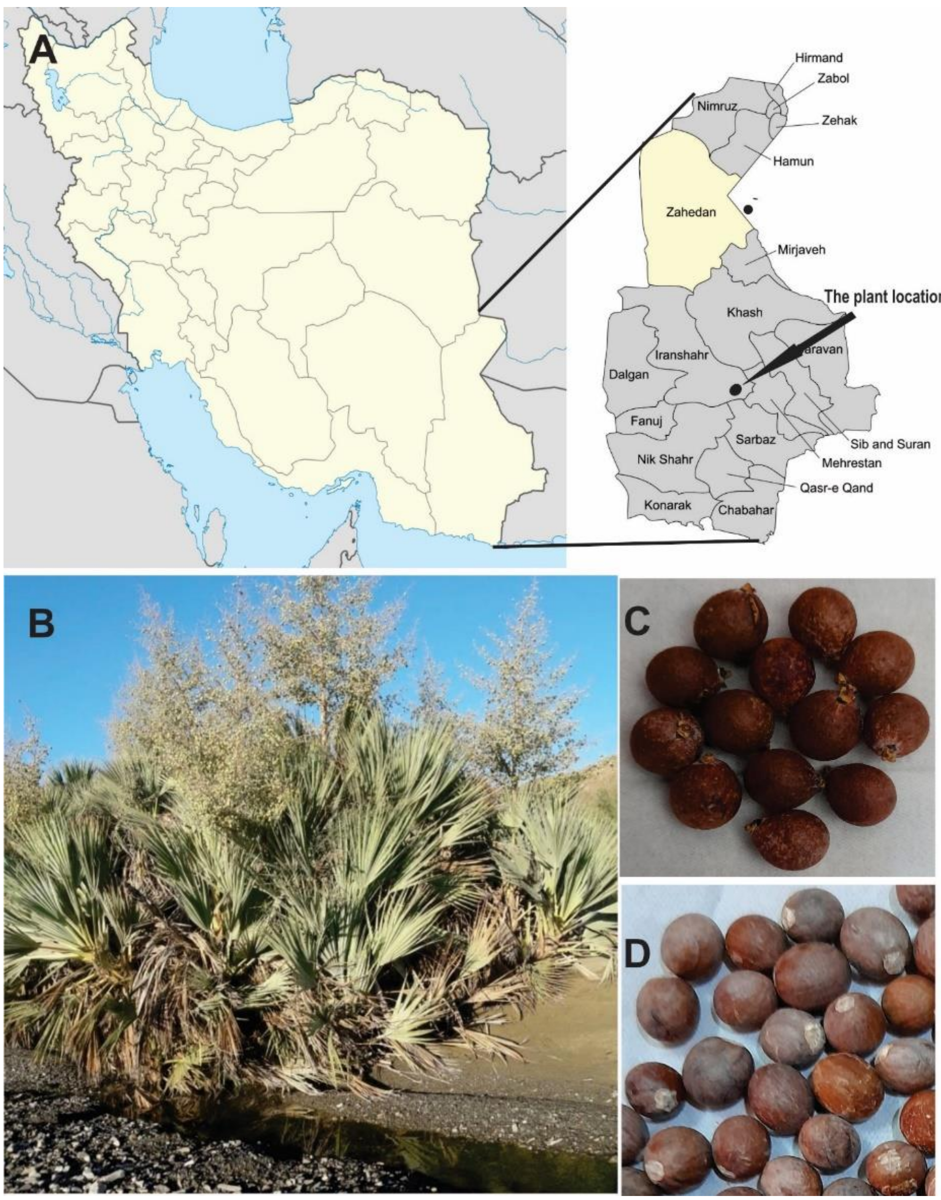

Figure 1. A. the locality of the examined population of *Nannorrhops ritchiana*. B. the habit of *Nannorrhops ritchiana*. C. the drupes. D. the seed morphology of the species

Table 1. The inclusion/exclusion criteria used to assess the biological properties of seed and fruit extracts of *Nannorrhops ritchiana*

Section	Inclusion	Exclusion
TPC	The absorbance of solutions containing methanolic extract, water, Folin-Ciocalteu and sodium carbonate at 760 nm	The total phenolic content as µg gallic acid equivalent (GAE) per gram of dried extract
TFC	The absorbance of solutions containing methanolic extract, methanol, aluminum chloride, potassium acetate and water at 415 nm	The total flavonoid content as µg QE per g of dry extract
AC	The absorbance of methanolic solutions containing extract and DPPH at 517 nm	Antioxidant capacity as IC_{50}
AE	The inhibitory effect of certain concentrations of the extracts against pathogens a specific population of pathogens	Inhibition zone diameters (IZDs) in mm

overnight. Then, the tubes were placed in the dark for another 24 hours. A volume of 0.5 mL of the seed and fruit extracts was severally mixed with 1.5 mL methanol (70%), 0.1 mL of aluminum chloride (100g/l), 0.1 mL of potassium acetate (1 M), and 2.8 mL distilled water. The mixture rested for 40 min at ambient temperature and pressure. Then, the absorbance of each solution was read in triplicate at 415 nm. This instruction was followed to record the absorbance of 0.5 mL of methanolic solutions of quercetin at various concentrations (50, 100, and 200 µg/mL). The next steps were taken according to those already explained in the previous section. The total flavonoid contents were reported as µg QE per g of dry extract.

Antioxidant Capacity

Using the DPPH free radical scavenging procedure, the IC_{50} values were quantified based on previous papers [22-24]. An amount of 1 ml of alcoholic extracts of the seeds and fruits of *Nannorrhops ritchiana* with concentrations of 250, 500, and 1000 $\mu g.ml^{-1}$ was added to 4 ml of freshly prepared methanolic solution of DPPH (0.004% w/v) in distinct tubes. After remaining in the dark for 30 minutes, the absorbance of each sample was read in triplicate, using a spectrophotometer apparatus. The blank sample consisted of 4 ml DPPH and 1 ml methanol. The equation: I% = [(blank absorbance-sample absorbance)/ (blank absorbance)]×100 was employed to calculate the inhibition percentage (I%), at 517 nm. Finally, the graph of concentration against the inhibition percentage was drawn and the 50% maximal inhibitory concentration (IC_{50}) was calculated when y equals 50. All tests were done in triple and represented as average.

Antimicrobial Experiments

The alcoholic extracts of the seeds and fruits of *Nannorrhops ritchiana* were prepared by soaking ten grams of each sample in 100 ml EtOH separately, followed by continuous shaking for 24 h. Then, the mixtures were passed through Whatman filter papers, and the filtrates remained at 37 ºC to evaporate. Solutions of all extracts and drugs were prepared in 10% DMSO and double-distilled water at initial concentrations of 10 $mg.ml^{-1}$ and 17.6 $\mu g.ml^{-1}$, respectively.

Gram-positive bacterial strains including *Staphylococcus aureus* (PTCC 1189), *Streptococcus pneumoniae* (PTCC 1240), *Rhodococcus equi* (PTCC 1633), *Bacillus subtilis subsp. Spizizenii* (PTCC 1023), *Enterococcus faeca* (PTCC 1778), Gram-negative bacterial strains including *Shigella flexneri* (PTCC 1234), *Proteus vulgaris* (PTCC 1079), *Salmonella typhi* (PTCC 1609) and fungal and yeast strains including *Aspergillus fumigatus* (PTCC 5009), *Fusarium oxysporum* (PTCC 5115) and *Candida albicans* (PTCC 5027) were purchased from the Persian Type Culture Collection (PTCC), Karaj, Iran. Kirby-Bauer disk diffusion susceptibility test was used to determine inhibition zone diameters (IZDs) [25]. The IZD values were reported as the average of triple separate tests. The inclusion and exclusion criteria of each section have been explained in detail in Table1.

All tests were performed in triplicate and reported as average ± SD (Standard Division). Data were analyzed statistically by ANOVA and Tukey's tests at a significance level of P Value < 0.05 using the SPSS statistical software (version 22).

3. Results

Determination of Total Phenolic and Flavonoid Contents

The total amounts of phenolic and flavonoid compounds in fruits and seeds of *Nannorrhops ritchiana* based on the Folin-Ciocalteu and aluminum chloride colorimetric protocols are presented in Table 2. The equation of the gallic acid calibration graph was

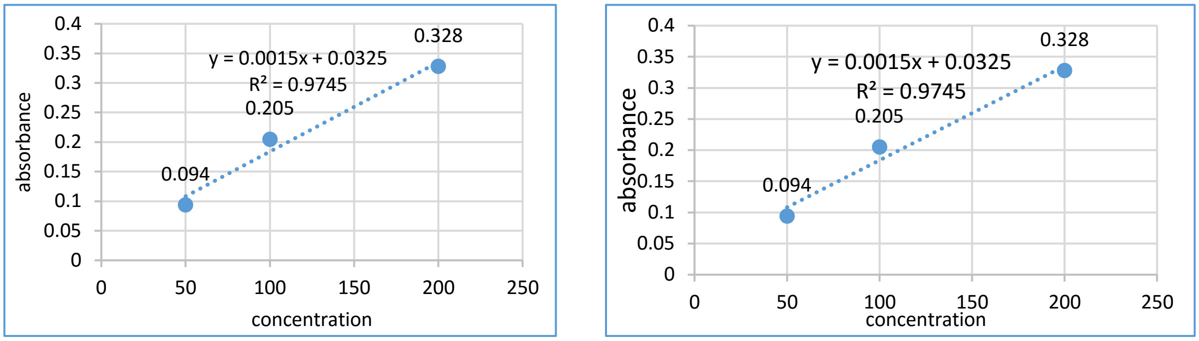

Figure 2. A. Calibration curve produced based on different concentrations of Gallic acid for calculating total phenolic compounds, B. Calibration curve produced based on different concentrations of quercetin for calculating total flavonoid compounds

Y=0.0015X-0.0325 (R^2=0.9745), according to which the total phenolic of the alcoholic extracts of seed and fruit of the examined plant were calculated (Table 2, Figure 2). The total flavonoid contents of the seeds and fruits were also calculated using the standard graph linear equation for quercetin (Y=0.004X+0.111, R^2=0.9639) (Table 2, Figure 2).

The values ranged for the total phenolic contents from 331.33 to 360.93 µg GAE/g dry weight and for the total flavonoid contents from 70.5 to 127.7 µg QE/g sample. In *Nannorrhops ritchiana*, the seeds contain slightly higher phenolic content compared with the fruits, while the flavonoid content of the fruit is much higher than that of the seed. Our results show that the seeds and fruits of *Nannorrhops ritchiana* are rich sources of phenolic compounds, proposing it for protection against free radicals-related diseases.

Scavenging Effects on DPPH Radical

The IC_{50} values of the alcoholic extracts of the seeds and fruits of *Nannorrhops ritchiana* and ascorbic acid (vitamin C), as a control, have been shown in Table 2. The IC_{50} value is inversely related to the antioxidant capacity of a substance [26,27]. Table 2 shows that the IC_{50} value for seed extract is lower than that of fruit indicating the higher antioxidant capacity in seeds compared to fruits of *Nannorrhops ritchiana.*

Also, the total antioxidant capacity of the seeds and fruits of *Nannorrhops ritchiana* is smaller than that of the control. The results of antioxidant capacity tests of the fruits and seeds of *Nannorrhops ritchiana* indicate a moderate to weak potential benefit for humans and animals.

Antimicrobial activity

The results of antimicrobial experiments of ethanolic extracts of seeds and fruits of *Nannorrhops ritchiana* on eleven bacterial and fungal pathogens have been presented in Table 3. The assessments were compared

Table 2. Total phenolic and flavonoid contents and antioxidant capacity (IC_{50}) of alcoholic extracts of the seed and fruit of *Nannorrhops ritchiana*

Samples	IC_{50} (µg.ml^{-1})	Total Phenolic content µg GAE /g	Flavonoids content µg QE/g
Fruit Ex.	204.34±7.59	331.33±8. 3	127.70±5.25
Seed Ex.	117.77±6.3	360.93±10.23	70.50±3.1
Vitamin C	3.94±0.43		

Table 3. Inhibitory activity of *Nannorrhops ritchiana* ethanolic extract against examined pathogens by disc diffusion method

Microbes Extracts/drugs	1240	1189	1023	1633	1778	1609	1079	1234	5009	5027	5115
Seed	7.82	NA	7.66	NA	8.24	NA	NA	9.12	8.10	8.54	9.04
Fruit	8.40	NA	8.60	NA	NA	NA	NA	7.32	7.20	NA	7.66
Ampicillin	15.91	17.03	34.18	15.72	28.61	30.24	20.40	30.63	-	-	-
Fluconazole	-	-	-	-	-	-	-	-	21.13	14.81	20.45

NA: Not applicable; -: Not measured.

with the antibacterial and antifungal effects of ampicillin and itraconazole, respectively, as standard drugs.

Table 3 shows that the extracts at initial concentrations could not inhibit the growth of *Staphylococcus aureus*, *Rhodococcus equi*, *Proteus vulgaris*, and *Salmonethella typhi*. No significant difference was observed in the antimicrobial effects of fruit and seed etanolic extracts, except that the seed extract was the only effective extract on *Enterococcus faeca* and *Candida albicans*. Also, *Fusarium oxysporum* was the most sensitive fungi to the ethanolic extracts of seeds and fruits.

4. Discussion

Data obtained from this study show that the seeds and fruits of *Nannorrhops ritchiana* are rich sources of phenolic and flavonoid compounds. The extract of seeds demonstrated higher phenolic those and antioxidant activity compared to that of fruits. Investigating the polyphenols quantification, anti-oxidant potential, and bactericidal and fungicidal effects of *Nannorrhops ritchiana* designated it as a valuable origin of natural polyphenols, with moderate antioxidant activity. Besides, it proposes *Nannorrhops ritchiana* as a fairly good agent to prevent the diseases caused by free radicals in humans and animals. Since many synthetic antioxidants are thought to harm the human body, much attention has been paid to natural antioxidants, especially phenols [28,29]. Many studies have shown a direct correlation between phenolic and flavonoid contents and the antioxidant potential of various plant organs. Even though the ethanolic extracts of the seeds and fruits of *Nannorrhops ritchiana* contain considerable amounts of phenolic and flavonoid compounds, only a moderate capacity for scavenging free radicals was seen in this study. This may be explained by the fact that phenolic compounds function as antioxidants non-specifically, and their ability to scavenge free radicals is determined, mainly but not strictly, by the number and location of phenolic hydroxyl groups [29-32]. Using DPPH free radical scavenging method, antioxidant properties of 70% methanol extract of *Nannorrhops ritchiana* leaves showed an IC_{50} value of 39.40 µg.ml^{-1} in comparison with ascorbic acid (IC_{50}= 1.8 µg/ml) [33]. This surprisingly indicates that the antioxidant potential of the leaves of *Nannorrhops ritchiana* is higher than those of fruits and seeds when compared with our results; however, we recommend reexam-ination of the leaves' antioxidant capacity.

The bioactive metabolites in medicinal plants fight with pathogens either by interfering with the biosynthesis of cell membranes, cell walls, and proteins or by blocking metabolic pathways or DNA replication [13]. Moderate antibacterial and antifungal activity were documented in this study for ethanolic extracts of seeds and fruits of *Nannorrhops ritchiana* against tested pathogens. The antifungal activity of 80% methanolic root extract of *Nannorrhops ritchiana* and its crude extracts including butanol, petroleum ether, ethyl acetate, and dichloromethane were evaluated using the agar tube dilution method to study the inhibitory property of the extracts against nine pathogenic fungi of humans, animals, and plants. The results proved a good to moderate antifungal activity of the root fractions, and the best antifungal effects were observed with crude petroleum ether extract fractions in the range of 67-80% inhibition on *Microsporum canis*, *Tricheophyton mentagrophytes*, and *Trichophyton longifusis* strains [34]. These results are in accordance with our findings that show moderate *in vitro* antifungal activity of the ethanolic extracts of seeds and fruits of *Nannorrhops ritchiana*. However, it should be noted that the antimicrobial activity of plant organs is both dose-dependent and strain-dependent [35,36]. The antibacterial and anti-fungal effects of ethanolic leaves extract of *Nannorrhops ritchiana* have also been assessed *via* disc and well diffusion methods, where inhibitory effects against all tested bacterial and fungal pathogens (IZDs of 5-21 mm) were observed from extracts with concentrations higher than 300 mg.ml^{-1} [37]. All extracts were especially effective on Gram-positive *Bacillus cereus* as well as Gram-negative *Pseudomonas aeruginosa* and *Klebsiella pneumonia* strains. Also, ethanolic fraction of aerial parts of *Nannorrhops ritchiana* showed only prominent antifungal activity against *Candida albicans* (62%), *Aspergillus niger* (66%), and *Microsporum canis* (60%), and moderate inhibition (56%) against *Staphylococcus aureus*, while other fractions were ineffective against tested microorganisms [38]. The results of this study were obtained in controlled conditions, which are not directly comparable to *in vivo* conditions. However, *in vitro* observations provide a foundation for *in vivo* and clinical studies. In addition, this study was conducted on a single population of *Nannorrhops ritchiana*, while geo-graphical or ecological populations of a plant may possess different genetic reserves and exhibit various degrees of biological properties. We highly reco-mmend more investigations on other populations of this valuable species to characterize a population with the highest amount of chemical composition, and the highest antioxidant and antimicrobial activity for *in vivo* studies.

5. Conclusion

Plant chemical compounds have received increasing attention due to their biological properties such as antioxidant and antimicrobial, for potential nutritional and therapeutic purposes. This paper evaluated the chemical composition, antioxidant potential, and antipathogenic activity of the seed and fruit ethanolic extracts of *Nannorrhops ritchiana*, an extensively used shrub by locals, to examine its potential for medicinal, nutritional, and industrial values. The fruit and seed ethanolic extracts of this plant contain high quantities of phenolic and flavonoid compounds, with moderate antioxidant capacity and moderate to good bactericidal and fungicidal activity against tested pathogens. These activities may be attributed to the high concentrations of phytochemicals, especially polyphenols, with the ability to scavenge free radicals or interact with cell membranes and walls of pathogens or other metabolic reactions. Further research is warranted to isolate *Nannorrhops ritchiana* active components and characterize their molecular structures in order to determine its potential usage for medicinal and nutritional purposes.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Author's contributions

Zahra Ebrahimnezhad performed the laboratory work and collected the data. Mehdi Dehghani contributed to the project idea, preparing the plant materials and writing the manuscript. Hamid Beyzaei contributed to providing the equipment for the experiments, data collection, and writing the paper. All authors have approved the final version of the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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