

In-Vitro* Evaluation of Phytochemical Profile and Pharmacological Potential of Methanol Derived, Natural Red Dye Extracted From Flowers of *Amaryllis Belladonna

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Abstract – Natural extracts are frequently admired in recent times for their safer pharmacological mode. A research effort was designed and aimed to detect the bioactive phytochemical constituents and to evaluate the pharmacological potential of *Amaryllis belladonna* flower red dye extract (*ABFRDE*). The methanol derived *ABFRDE*, was a potent source of phytochemicals such as alkaloids (6.16%), flavonoids (0.12%), tanins (0.031%) and phenols (17.951%). The *ABFRDE* was tested against gram-negative (*Escherichia coli*) and gram-positive (*Micrococcus leutus*, *Bacillus subtilis*, *Staphylococcus aureus*) bacterial strains and fungal strains (*Aspergillus niger*, *Aspergillus flavous*, *Aspergillus fumigatus*). Highest inhibitory activity against *M. luteus* (6mm) & *S. aureus* (5mm) at 15mg/ml. *M. luteus* (5mm) and *S. aureus* (4.5mm) was achieved at 7.5 mg/ml of the *ABFRDE*. The inhibition in growth of all the tested bacterial strains was minimum at 1.875 mg/ml of the *ABFRDE*. Maximum antifungal potential against *Aspergillus fumigatus*, *Aspergillus flavous*, *Aspergillus niger* was recorded at highest concentration (15mg/ml) of the *ABFRDE*. The free radical scavenging action of the *ABFRDE* was performed as % inhibition of DPPH (1, 1-diphenyl-2-picryl-hydrazyl) free radicals. The DPPH radical scavenging potential of the various concentrations of the *ABFRDE* can be ranked as 5 mg/10 ml > 4mg/10 ml > 3mg/10 ml > 2mg/10 ml > 1mg/10 ml. We suggest further investigation of *ABFRDE* for identification of its active ingredients.

Keywords – *Amaryllis belladonna*, antimicrobial, antioxidant, 1, 1-diphenyl-2-picrylhydrazyl

1. Introduction

Amaryllis belladonna is commonly called belladonna lily. Belladonna means “beautiful woman” the plant was so named by Linnaeus because of its attractive flower. It’s also some time known as naked lady by North American because the plant produces flower on the top of a bare stalk. In South Africa, it’s traditionally called as March lily, because its blooming period is from March to April [1]. *Amaryllis belladonna* belongs to family Amaryllidaceae. It is perennial and can be propagated through bulbs [2]. Plants of the family Amaryllidaceae are much therapeutic importance [3].

Plants have been served as green medicines since very ancient time [4]. These medicines were initially applied as crude drugs in the form of infusions, tinctures, decoctions, teas, poultices, powders, and other herbal preparations [4].

Medicinal plants distinguish a significant health and financial constituent of biodiversity and also conservation and sustainable use [5]. Plant processed in different formulations can be used to treat an extensive variety of diseases and may explain brilliant results against different pathogens [6]. They can be described as natural factories of phytochemicals like Phenolics acid, terpenoides and alkaloids.

Phenolics acid offers antioxidant, cytotoxic, antiulcer, anti-inflammatory, antitumor and antispasmodic actions while terpenoides and alkaloids beside their other medicinal uses are inhibitory to pathogenic microbes [7-

10].

Plant-derived natural dyes are easily extracted, completely biodegradable, and readily available. Many natural dyes, including carotene, cyanine, tannin, anthocyanin and chlorophyll, have been studied. Carotenoids, which are responsible for the coloration of many plants and fruits, possess antioxidant activity due to their reactivity with singlet oxygen and oxygen free radicals [11]. As important nutrients, carotenoids may help to delay or avoid oxidative damage and offer protection against cancer, cardiovascular disease, eye diseases, atherosclerosis, and the aging process [12].

Plants of the family *Amaryllidaceae* being equipped with phytochemicals, have been used as folk remedies [13]. *Amaryllis belladonna* has been applied in Vietnamese traditional medicines, as a remedy against tumors [14]. Medicinal plants comprises of phytochemical constituents which have specific physiological action on the human body and these constituents include terpenoids, alkaloids, tannins, steroids, carbohydrates, flavonoids and other bioactive compounds [15]. The most significant bioactive compound isolated from the plants of family *Amaryllidaceae* is rutin, which is a flavonoid [16]. Rutin has been reported to be potent against inflammations, ROS, carcinoma and cardiovascular diseases [17].

Amaryllis belladonna has been reported to be potent against *Plasmodium gallinaceum*. The flowers of *Amaryllis belladonna* have been recognized for their

Alkaloid profile [18]. A single plant can be made in to different formulations which can be used to defeat different pathogenic strains of microbes. Research work has revealed the antifungal potential, particularly the antiyeast activities of *Amaryllis belladonna*, *Crinum macowanii* and *Crinum moorei* [6].

No effort has been made for the evaluation of biological activities of the *ABFRDE*. The present investigation was aimed to determine the

- (a) Phytochemical status of *ABFRDE*.
- (b) Antimicrobial potential of *ABFRDE*.
- (c) Antioxidant potential of *ABFRDE*.

2. Materials and Methods

2.1. Collection of flowers of *Amaryllis belladonna*

The flowers of *Amaryllis belladonna* were collected from Botanical Garden, University of science and technology Bannu KPK, Pakistan. The flowers were shade dried and ground to obtain fine powder.

2.2. Preparation of Methanolic extract

10 g of *Amaryllis belladonna* flower powder was suspended in 300 ml of Methanol for 48 hours, followed by filtration of the suspension using filter paper (Whatman No.1). Solvent was then evaporated at 45°C for 72 hours to obtain the dye extract. The total yield of dye extract was 15.42%.

2.3. Phytochemical Analysis

Standard experimental protocols were adopted for screening of bioactive phytochemical constituents in the *ABFRDE*.

2.4. Qualitative Analysis

Maeyer's and Dragendorff's Reagents were prepared for the qualitative analysis.

2.4.1. Test for Alkaloids

0.5-0.6 gram of the *ABFRDE* of the plant under investigation was mixed with 8 ml of 1% HCl. The mixture was warmed and filtered. 2 ml of the filtrate was mixed separately with both Maeyer's and Dragendorff's reagents and the alkaloid content was guessed from whether the presence or absence of precipitate.

2.4.2. Test for Terpenoids (Salkowski Test)

5 ml of *ABFRDE* was added in to a test tube containing 2 ml of chloroform followed by the careful addition concentrated H₂SO₄ (3 ml). A layer of the reddish brown coloration was observed at the interface thus indicating the presence of terpenoids.

2.4.3. Test for Flavonoids

A sample of 0.5 gram of the *ABFRDE* was mixed with petroleum ether to eliminate fatty materials (lipid layer). The residue was suspended in 20 ml of 80% ethyl alcohol and filtered. The filtrate was further processes as follows.

- (i) 3ml of the filtrate taken in a test tube was let to react with 4 ml of 1% aluminium chloride in methanol.

Appearance of yellow coloration confirmed the presence of flavonols, flavones and chalcones.

- (ii) A sample of 3ml from the filtrate was mixed with 4ml of 1% potassium hydroxide in a test tube. Appearance of dark yellow coloration revealed the presence of flavonoids.

2.4.4. Test for Tannins

A sample of 0.25 gram from the *ABFRDE* added into 10 ml distilled water was shaken and filtered. 1% iron chloride (FeCl₃) solution prepared in distilled water was added into the filtrate. Presence of tannins was confirmed from the appearance of intense green, purple, blue or black color.

2.5. Quantitative Analysis

2.5.1. Alkaloids determination using Harborne (1973) method

Calculated quantity of the *ABFRDE* was dissolved in 200 ml of 10% acetic acid. and allowed to stand for. 4 hrs. After filtration the solution was concentrated to 1/4th of its original volume while heating it gently in a water bath. The concentrated ammonium hydroxide was added dropwise until the precipitation was completed. The whole solution was allowed to settle. The precipitate was collected carefully from the solution and was washed with dilute ammonium hydroxide and filtered. The residue (alkaloid) was weighed and its percentage was calculated after complete dryness.

2.5.2. Tannin determination by Van-Burden and Robinson (1981) method

The *ABFRDE* (500 mg) was added into 50 ml of distilled water and kept in mechanical shaker for 1 hr. It was then filtered into a 50 ml volumetric flask.. The filtrate (5ml) was sucked through pipette, was poured into a test tube. The filtrate was mixed with 2 ml of 0.1 molar FeCl₃ in 0.1 molar HCl and 0.008 molar potassium Ferro cyanide. The absorbance was measured at 120 nm within 10 minutes.

2.5.3. Determination of total Phenols by Spectrophotometric method

The *ABFRDE* (0.001gm) was allowed to fully dissolve in 10 ml of methyl alcohol in a test tube. The 125 µl of the solution was mixed with 125 µl folin reagent and 500 µl of distilled water. The solution was kept for 5 min. Distilled water was added to the solution to raise its volume to 3 ml. The 1.25 ml of sodium bicarbonate aqueous solution (7%) was added to the sample. The sample was stand till 90 minutes and its optical density was taken at 760 nm with the help of spectrophotometer. Methyl alcohol was used as blank. The readings were compared with a standard curve of various gallic acid and the contents of total soluble phenolics was expressed as mg galic acid eq./gm d.w.

2.5.4. Flavonoid determination by the method of Bohm and Kocipai-Abyazan (1994)

The *ABFRDE* (10 gram) was dissolved in 100 ml of

80% aqueous methyl alcohol at 25 °C. The solution obtained was passed through filter paper (Whatman # 42). The filtrate was poured into a crucible and was allowed to evaporate over a water bath. The percentage quantity of the residue (flavonoids) was calculated.

2.6. Antibacterial assay

Agar well diffusion method (Well diffusion method) was adopted for the evaluation of antibacterial activity (Kavanagh, 1963; Leven, 1979). The *ABFRDE* was tested against four bacterial strains namely, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Micrococcus luteus*. The strains were cultured on nutrient agar media in four separate petri plates. Five wells were made in the Agar medium of each petri plate designated as A, B, C, D, and E. 40µL of 1mg/10ml isoniazid (antibiotic) solution was added in one well, while 40µL of 15mg/ml, 7.5mg/ml, 3.75mg/ml and 1.875mg/ml of the *ABFRDE* solution was added separately into each of the remaining four wells. All the solutions were made in dimethyl sulfoxide (DMSO). The strains were incubated at room temperature for 24 hours. After 24 hours, the area of inhibition was measured.

2.7. Antifungal assay

The Agar Tube Dilution method was followed for antifungal assay [19]. The *ABFRDE* was tested against three pathogenic fungal strains, the *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus fumigatus*. The fungal strains were first refreshed on Sabouraud Dextrose Agar (SDA) medium. Four different concentrations of *ABFRDE* were prepared in DMSO, i.e. 10mg/ml, 5mg/ml, 2.5mg/ml and 1.255mg/ml. Slant were prepared by pouring 3ml agar solution (composition 6.5grams/100ml) and 50µl of *ABFRDE* solution into test tubes. A total of 33 slants were prepared, i.e. 6 for each concentration, 6 for terbinafine (control) and 3 containing only the medium. After solidification of medium, the strains were inoculated and incubated at 37 °C. After one week, the growth zone was measured with the help of a measuring scale.

The % inhibition was calculated as:

% inhibition of fungal growth = $[(100 - \text{linear growth in test sample in mm}) / (\text{linear growth in control in mm})] \times 100$ [19].

Table 1: Qualitative Analysis of *ABFRDE*

Sample code	Alkaloids	Flavonoids	Tannins	Phenols
<i>ABFRDE</i>	++	+++	++	+++

- = not present, + = present, ++ = moderate, +++ = highly present

Table 2: Quantitative Analysis of *ABFRDE*

Sample Code	Alkaloids (%)	Flavonoids (%)	Tannins (%)	Phenols (mg gallic acid eq./gm dry weight)
<i>ABFRDE</i>	6.16	0.12	0.031	17.951

2.8. Antioxidant activity (DPPH free radical scavenging assay)

The scavenging activity against DPPH (1, 1-diphenyl-2-picryl-hydrazyl) free radicals was determined by different testing concentrations [20]. Methanolic solution of DPPH was prepared by dissolving 3mg DPPH in 50ml methanol. We found the optical density using spectrophotometer at the wavelength of 517nm. It was 0.940. 2.8ml of this solution was added to 200 µl of the solution of all extracts in methanol at different concentration (1mg/10ml, 2mg/10ml, 3mg/10ml, 4mg/10ml, and 5mg/10ml). We used Ascorbic acid was used as the reference (control). Lower absorbance values of reaction mixture inferred higher free radical detoxifying activity. All the tests were performed in triplicates to obtain precise data.

The scavenging activity was projected on the basis of percentage of DPPH radical scavenged using the following formula:

Scavenging % = $[(\text{control absorbance} - \text{sample absorbance}) / (\text{control absorbance}) \times 100]$

2.9. Statistical analysis

Standard deviation tests were applied for statistical analysis of data.

3. Results and Discussion

3.1. Phytochemical analysis

The *ABFRDE* showed the presence of alkaloids, flavonoids, Tannins and phenols. The flavonoids, and phenols were detected in higher concentration (Table 1).

The *ABFRDE* was studied quantitatively for the chemical constituents. Alkaloids (6.16%), flavonoids (0.12%), tannins (0.031%) and phenols (17.951 mg gallic acid eq./ g D.W) were present in concentration as shown in (Table 2 and Fig. 1). The results showed that the *ABFRDE* was a good source of natural alkaloids, flavonoids, tannins and phenols.

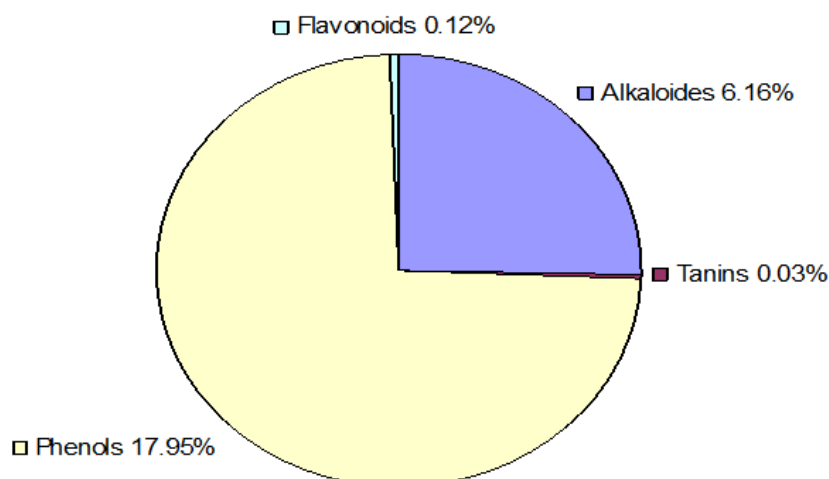
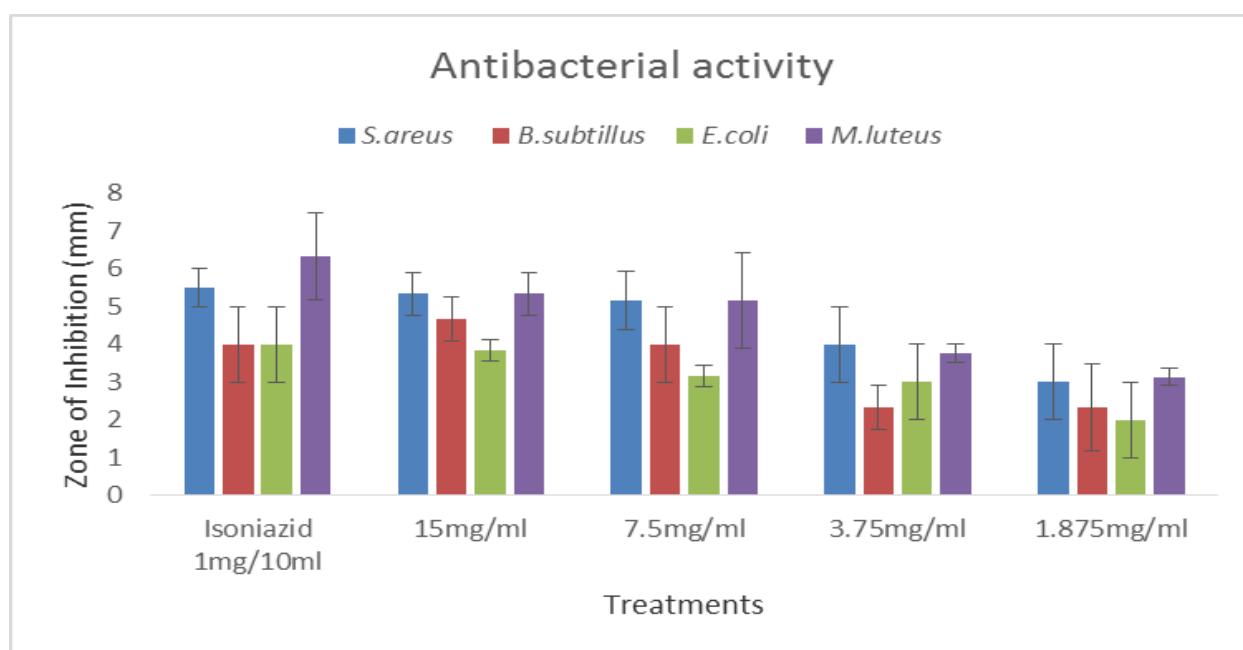


Fig. 1. Percentage of phytochemicals

3.2. Antibacterial activity

Various concentrations of *ABFRDE* were evaluated against four bacterial strains (*Escherichia coli*, *Micrococcus luteus*, *Bacillus subtilis*, and *Staphylococcus aureus*). Highest inhibitory activity against both *M. luteus* & *S. aureus* (5.33mm) at 15mg/ml. An inhibitory zone diameter of (5.1mm) against both *M. luteus* and *S. aureus*

was achieved at 7.5 mg/ml of the *ABFRDE*. The minimum antibacterial activity against *B. subtilis* and *E. coli* was observed at 1.875mg/ml. All the tested bacterial strains were highly susceptible to Isoniazide used as control & exhibited maximum zones of inhibitions (Fig. 2). Statistical treatment of the data showed variability among values of replica as relevant from standard deviation bars.

Fig 2. Antibacterial activity of *ABFRDE*.

3.3. Antifungal Activity

Fig. 3 showed maximum antifungal activity against *A.niger*, *A.flavus*, *A.fumigatus*, at highest concentration of *ABFRDE*. The antifungal activities of *ABFRDE* were studied at different concentrations (10, 5, 2.5 and 1.5mg/ml). The extract showed antifungal activity with inhibition zone of 52, 27.3, 42.6 and 35.33mm, at 10, 5,

2.5 and 1.5mg/ml respectively against *A. flavus*. A maximum inhibition zone diameter of 40.3mm was measured against *A. niger* at 2.5mg/ml of the extract. The growth of *A. fumigatus* was inhibited with zone diameter of 58mm at 10mg/ml of the extract concentration (Fig. 3). Standard deviation bars indicate closeness among the values of replica.

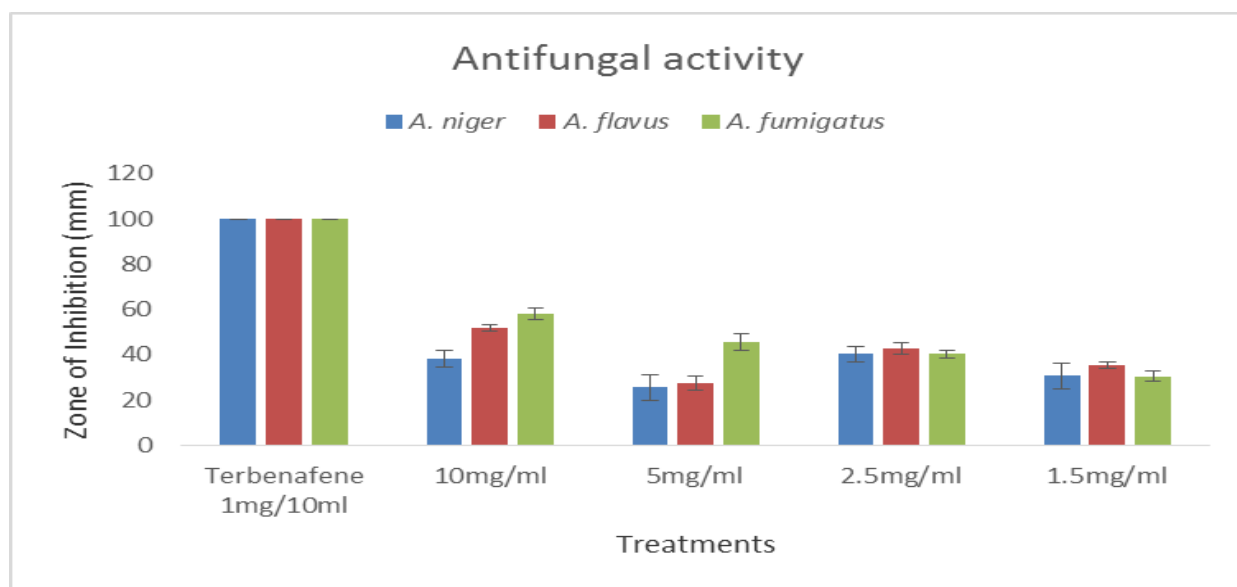


Fig 3. Antifungal activity of *ABFRDE*. Data represent mean of three replicate.

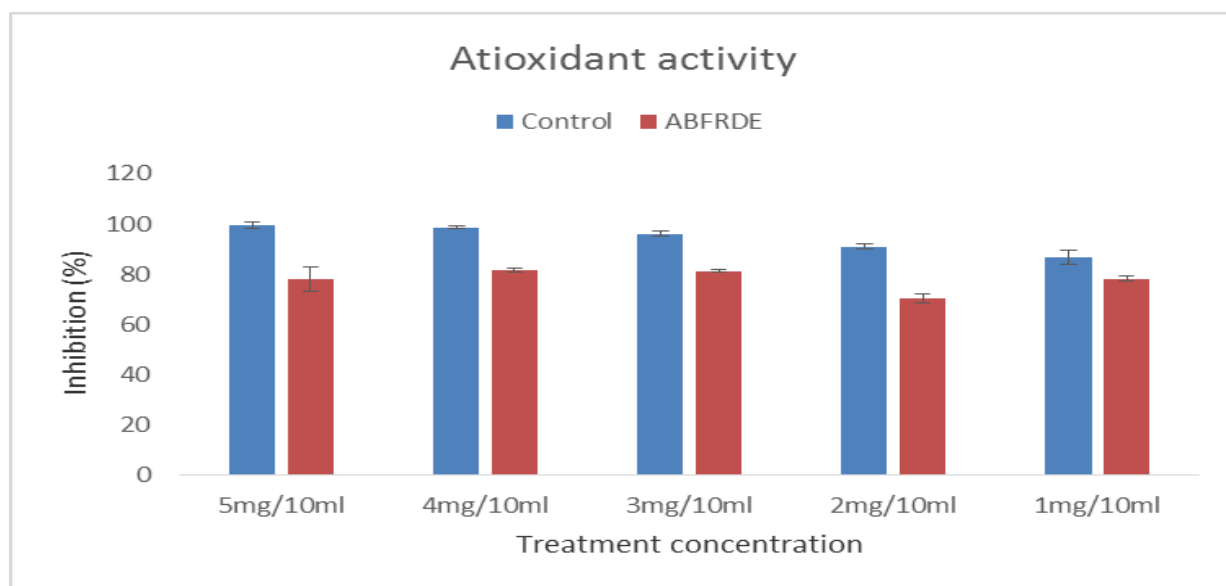


Fig 4. Antioxidant activity of *ABFRDE*.

3.4. Anti-oxidant activity

(Fig. 4) showed that the DPPH radical scavenging activity of the various concentrations of the *ABFRDE* can be ranked as 5 mg/10 ml > 4mg/10 ml > 3mg/10 ml > 2mg/10 ml > 1mg/10 ml. The higher antioxidant activity of the *ABFRDE* might be due to presence of the most

important biologically active flavonoid rutin. (Fig. 4).

The present investigation was based on the pharmacological assessment of *ABFRDE*. The dye was extracted in methanol by common maceration technique. The *ABFRDE* was tested evaluated for its antimicrobial and antioxidant potential. Medicinal plants or their parts are plant derived drugs, applied for medication in various

health care systems. Plant derived drugs constitute a kind of traditional medication practiced globally [4].

Tallini et al., 2017 [2] have isolated more than 20 different kind of alkaloids from the bulbs of *Amaryllis belladonna* by Gas chromatography-mass spectrometry (GC–MS) studies. He et al., 2015 have reported excellent biological activities of alkaloids isolated from plants of family Amaryllidaceae. Havelek et al 2014 [21] have demonstrated phytotherapeutic potential of phytochemicals obtained from plants of Amaryllidaceae family. Yu-Bin et al., 2013 [22] have published their article on anti-tumor, antiinflammatory, antibiosis, antiviral and antimalarial activities of *Lycoris radiata*, *Crinum asiaticum* and *Curculigo orchioides*. Babashpour-Asl et al., 2017 [23] have purified Nerinine and Homolycorine from the bulbs of *Galanthus transcaucasicus*. Evidente, et al., 2004 [24] have demonstrated excellent antifungal activity of Amaryllidaceae alkaloids. Sener et al., 2003 [25] have published the antimalarial activity of extracts of phytochemicals derived from *Amaryllidaceae* plants. Katoch and Singh 2015 [26] have reported excellent pharmacological activities of the Genus *Zephyranthes* of family *Amaryllidaceae*.

The pharmacological activities of natural sources can lead to the exploration of novel antimicrobial constituents that can be applied to selectively target infectious diseases of plants and animals as well. During present investigation the *ABFRDE* exhibited antibacterial and antifungal activities against tested bacterial and fungal strains. The application of plants as folk medication is a well-established practice in rural areas of many developing parts of the globe. Sandhu and Heinrich, 2005; Mitscher et al., (1987) [27, 28] have postulated that higher plants are the potent sources of novel antibiotic compounds. Natural antibiotic drugs can be obtained from plants, animals and microbe themselves. The limitations of the available array of drugs today have compelled the researchers to search obtain new therapeutic agents from natural sources [29]. In vitro studies of a large array of phytochemical constituents belonging to different classes have shown their inhibitory potential against microorganisms [30]. Some plant derived extracts have been indicated to be active against both Gram positive and Gram negative strains of bacteria [31]. Presently, different chemical compounds of phyto-origin are applied as active remedies [32]. Presently there is no single plant derived antibacterial chemical entity used clinically. Different compounds have been used for this purpose mostly isolated from medicinal plants [32].

The *ABFRDE* exhibited antioxidant activity measured as % inhibition of DPPH free radicals. Oxidative stress is believed to be a major contributor to the pathogenesis of a number of chronic diseases, and it is for this reason that antioxidant behavior is one of the most commonly determined biological activities in extracts of plants [33]. The results of the present investigation established that *ABFRDE* possess some natural biological compounds with profound antioxidant activity. The higher antioxidant potential of the *ABFRDE* reveals that it can be a better alternative to synthetic antioxidants for various applications such as herbal medicines. As the *ABFRDE* exhibits antioxidant activity, therefore it must be tested for

cytotoxic and anticancer activities. The plant extracts exhibiting good antioxidant potential are usually effective anticancer agents [34].

4. Conclusion

The *ABFRDE* exhibited antibacterial (*Escherichia coli*, *Micrococcus leutus*, *Bacillus subtilis*, *Staphylococcus aureus*) and antifungal (*Aspergillus fumigatus*, *Aspergillus flavous*, *Aspergillus niger*) activity. The DPPH free radical scavenging activity was higher at 5 mg/10 ml. The results of present study established that *ABFRDE* is useful for medicinal point of view and further works are needed to isolate and purify the bioactive compounds present in it.

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