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Research Article
Evaluation of Phytochemical, Antioxidant and Anti-inflammatory Properties of near Endemic *Aloe dhufarensis*

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Abstract

Background and Objective: *Aloe dhufarensis* is a local endemic medicinal herb used for centuries to treat diabetes, headache, fever, skin disorders and wound healing by the local people of Oman. The present research was initiated to substantiate the ethno-medicinal uses of this plant by assessing the phytochemicals and evaluating the antioxidant, anti-inflammatory and anti-microbial activity of *Aloe dhufarensis*. **Materials and Methods:** Dried, finely grinded leaves of *A. dhufarensis* were extracted with methanol for phytochemical analysis using standard protocols and several phytochemical such as saponins, alkaloids, terpenoids, flavonoids, steroids, tannins, glycosides, anthraquinones, phytosterol, coumarins and carbohydrates were detected in the extract. **Results:** Extracts displayed significant antioxidant property, exhibiting a potent DPPH scavenging activity with IC_{50} value of 83.46 μg mL^{-1} and a strong hydrogen peroxide scavenging activity with an IC_{50} value of 289.786 μg mL^{-1}. Substantial inhibition of protein denaturation and anti-proteinase activity were recorded. Considerable antibacterial activity was observed against all 4 studied strains with the highest antibacterial activity recorded against the *E. coli*. **Conclusion:** The results reveal that *A. dhufarensis* possess a wide spectrum of phytochemicals that have significant pharmacological properties and substantiate the support to the scientific basis for the traditional use of this ethnomedicinal herb.

Key words: *Aloe dhufarensis*, anti-inflammatory, anti-oxidant, total phenols, phytochemical analysis

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Oman is a spectacular country in Arabian Peninsula in terms of biodiversity and is home to 78 endemic and 48 near-endemic plant species, with substantial number of these species being found in SouthernDhofarregion[1]. This refugium harbors many important medicinal plants, one of them is *Aloe dhufarensis* Lavranos, a threatened species, near endemic to Dhofar province and neighbouring Eastern Yemen. It is the most xerophytic of all the Aloe species found in Oman. Locally called Subr or Sakkal, is found growing in dry water courses, gravel banks and on high plateau areas at an altitude from 1200-1400 m on Jabal al Qamar range of Al Qarah Mountains in Dhofar, forming an endemic shrub land community along with *Sedderaglomerata* and perennial small shrubs *like Pulicariaargyrophyllum* subsp. *oligophylla* and *Portulacadhofarica*[1].

This evergreen succulent perennial is a stem less solitary rosette about 1.5 m tall with 10-20 basal upright pale grey lance shaped, slightly incurved waxy leaves. The leaves are lightly spotted and mainly toothless with few whitish teeth seen in young plants. Tall branched inflorescence around 90 cm, bearing striking coral red pendulous flowers in conical racemes, blooms during early spring till June. Being xerophytic the leaves develop large thin walled water storage parenchyma cells containing a viscous gel in the inner pulp region. The outer pulp region contains an orange yellow sap/latex.

Many medicinal uses have been ascribed to this species in the traditional herbal medicine, the juice extracted from the succulent leaves has been used to treat headaches, diabetes and for wounds healing[2,3]. The pharmacological effects exhibited by the plants are manifestations of presence of active secondary metabolites which impart biological activity to the plant. Substantial evidence exist where therapeutic potential of a medicinal plant is associated with the physiological effect of phytochemicals like flavonoids, phenols, alkaloids, anthraquinones, steroids and terpenoids[4,5]. Aloe genus is specifically rich in secondary metabolites like anthraquinones, flavonoids and terpenoids[3,6].

Phenolic compounds like phenolic acid and flavonoids have bioactive effects due to their high antioxidant ability[7]. Phenolic compounds, antioxidant potential depends upon the degree of hydroxylation and polymerization as the hydroxyl functional group inhibits oxidation by neutralizing the unstable reactive free radicals by donating electrons and by chelating the metal ions[8,9].

Anthraquinones, the other class of poly phenolic compounds in polyketide group are widely used in medicinal chemistry due to their wide range of therapeutic potential exhibiting laxative, diuretic, antidiabetic[10], antioxidant[11], anticancer[12], anti-inflammatory[13], antibacterial[14] and antifungal properties. Anthraquinones are also associated with inhibition of Advanced Glycation End-products (AGEs), which are usually associated with chronic complications of diabetes mellitus. The main anthraquinones found throughout the genus Aloe are emodin and aloe emodin[6].

Terpenoids mainly triterpenes lupeol a pharmacologically very active terpenoid and tannins which are another group of antioxidant polyphenols are also commonly found in Aloe genus[15], though alkaloids the analgesic and the cardiac stimulants are found only in few species of Aloe genus[16].

Chemical composition specifically the secondary metabolites of any plant species reflects the intricate interaction between the species and its surrounding environment, geographical conditions, the type and composition of soil and thus helps the species to survive better in that particular habitat[17,18]. Under arid xeric environmental conditions, soaring temperatures and extreme water stress induces oxidative stress and under such conditions xerophytic plants elicit their adaptive survival strategies via production of increased amount of secondary metabolites like flavonoids and phenolic acids[19]. Such arid zones are ecologically exclusive, displaying significant endemism, where species thrive under specialized ecological niches called "Refugia", restricted to certain geographic ranges and these hotspots, under the influence of specialized phytoclimate accumulate some unique secondary metabolites as their adaptive response[20,21].

The *A. dhufarensis* is found along the xeric habitats of North facing slopes of escarpment at the high plateau of Jabal Qamar range of Al Qarah Mountains[22].

Though one of the most important medicinal plant of Dhofar region with wide implications in traditional medicine, no previous detailed quantitative analysis to authenticate the pharmacological effects of *A. dhufarensis* is reported. Further with the other commercially important species of Aloe standing tall in terms of global trade there is an compelling need to explore the vast commercial value of locally used aloe species. As this species for centuries has been used by locals for remarkable health benefits, the research aimed to validate the therapeutic potential of the important medicinal species *Aloedhufarensis*, thus the present research was initiated to estimate the phytochemicals, antioxidant, anti-inflammatory and anti-microbial activity as influenced by the local xeric environmental conditions in *Aloe dhufarensis*.

MATERIALS AND METHODS

Study area: The present study was conducted from 14th February-20th December, 2018 in the Department of Biology, College of Science, Sultan Qaboos University, Muscat, Oman.

Plant material: Healthy *Aloedhufarensis*, plants growing wild were collected on 6th March, 2018 from Jabal Al Qamar Mountains, Dhofar, Oman. The temperature recorded was 28.4±2°C. The collected plants were identified and compared with the herbarium sheets of specimen at the Herbarium, Life science unit, Sultan Qaboos University, Oman.

Plant extract: Fresh leaves were washed, chopped into smaller pieces and kept under shade (25°C) for 2 weeks to dry completely. Dried leaves were ground into a fine powder and 100 g of dried leaf powder was mixed with 300 mL of pure methanol (99.9%) and kept in a temperature-controlled shaker for 24 hrs. The residues after the first extraction were subjected to second and a third extraction and filtrate evaporated to complete dryness using a rotary evaporator. The residue was dissolved in dimethyl sulfoxide to yield 100 mg mL^{-1} of extract.

Antibacterial activity: The following ATCC cultures of *Escherichia coli* (ATCC 9637), *Pseudomonas aeruginosa* (ATCC 10231), *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 29213) were used for the antimicrobial activity.

Disc diffusion method: Antibacterial efficacy was estimated following disk diffusion assay[23]. Nutrient agar plates were inoculated with bacterium strain and cultured overnight at 37°C. Sterilized filter paper discs (6 mm) soaked in 100 µL of the test solution were laid on the inoculated agar plates. DMSO was employed as a negative control and Ampicillin (1 mg $discG^{1}$) was used as a positive control. The inoculated agar plates with discs were incubated at 37°C for 24 hrs and later diameter of the clear zones, measured in millimetres. Experiment analysis was done in triplicate, mean, standard deviation and standard error were calculated.

Minimum Inhibitory Concentration (MIC): The Minimum inhibitory concentration was evaluated through the microtiter test[24]. Based on preliminary screening in disc diffusion method, methanolic leaf extracts that displayed effective anti-bacterial activity were tested for MIC. Leaf extract was serially diluted, in the range of 10-0.078 mg mL^{-1}, then 100 µL of different concentrations (10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15655 and 0.078 mg mL^{-1}) were loaded in each well of 96-well microtiter plates and kept to incubate for a period of 24 hrs at a temperature of 37°C. The lowest concentrations of the A. *dhufarensis* leaf extract (µg mL^{-1}) which inhibited the bacterial growth, with no turbidity observed are defined as MIC.

Phytochemical screening: Phytochemicals like saponins, alkaloids, terpenoids, flavonoids, steroids, tannins, glycosides, anthraquinones and coumarins in *A. dhufarensis* was evaluated using the standard test methods described below[25-27]. All the tests were performed in triplicate.

Test for saponins (frothing test): The 0.5 mg extract and 10 mL distilled water were shaken strongly in a tube. The formation of foam or froth which persisted even after 30 min of letting the mixture to stand, indicated the presence of saponins[25].

Tests for flavonoids (the shinoda test): In this test 5 mL leaf extract, 4 strips of magnesium filings with few drops of concentrated HCl were mixed. The appearance of reddish color indicated the presence of flavonoids[26].

Test for tannins (Ferric chloride test): The 0.5 g extract in water (10 mL) was boiled and filtered to that few drops of 10% ferric chloride solution was added, a blackish-blue colour indicated gallic tannins while green-blackish colour indicated catechol tannins[26].

Test for Terpenoids (Salkowski test): Leaf extracts (1 mL)+chloroform (0.5 mL)+few drops conc. sulphuric acid, emergence of yellow colour ring which changed into a reddish-brown color after few min, establishes the presence of terpenoids[25].

Test for steroids (Liebermann-Burchard's or Acetic anhydride test): The 0.5 g of *A. dhufarensis* leaf extract and 10 mL anhydrous chloroform were mixed and filtered, to this acetic anhydride (2 mL) followed by conc. sulphuric acid (2 mL) was added. Change in color from violet to blue or green established the occurrence of steroids[25].

Test for phytosterol: Alcoholic potassium hydroxide was refluxed through the leaf extract till the complete saponification, to this H_2O was added and using ether as a solvent, this mixture was extracted in a water bath, ether was

completely evaporated and to the residual aqueous layer, dilute acetic acid (few drops), acetic anhydride (3 mL) and conc. H_2SO_4 (few drops) were added. Formation of bluish green color indicated the existence of phytosterol[27].

Test for glycosides (Cardiac glycosides (keller-killiani test): Glacial acetic acid (2 mL), ferric chloride solution (one drop) were mixed with *A. dhufarensis* leaf extract (5 mL) and to this mixture conc. sulphuric acid (1 mL) was added drop by drop. Emergence of a violet ring confirms the occurrence of cardiac glycosides[25].

Test for alkaloids (The Mayer's test): In this test mercuric chloride (1.36g) and potassium iodide (5 g) was dissolved in 100 mL water to prepare fresh Mayer's reagent. Dropwise 1 mL of the reagent was mixed with 1 mL of methanolic leaf extract. Precipitate formation (cream colored) indicated occurrence of alkaloids[26].

Test for coumarins: One gram extract in a tube was moistened with H_2O, a filter paper moistened with dilute NaOH was firmly positioned on the rim of the tube. The tube was heated for 15 min in a water bath and finally, filter paper was pulled out and ultra-violet light was directed towards it, a yellowish-green luminosity confirms the occurrence of coumarins[25].

Test for quinones: Appearance of a red color after adding conc. sulphuric acid (1 mL) to 1 mL of the leaf extract demonstrated the occurrence of quinones[26].

Determination of total phenolics (Folin-Ciocalteu reagent): Folin-Ciocalteu reagent assay was applied to ascertain the total phenolic content spectrophotometrically[28]. 0.5 mL of methanolic extract (100 μg mL^{-1}) was mixed with 2.5 mL of Folin-Ciocalteu reagent and 7.5 % Na_2CO_3 (2.5 mL), the test tube was covered with paraffin and then kept for 30 min under dark conditions. Absorbance was read at 765 nm. Solution of Folin-ciocalteu, Na_2CO_3 and methanol acted as blank and reference standard was gallic acid. Assessment of total phenolic content was made using the linear equation of the calibration curve, expressed as milligrams of gallic acid equivalents per gram (mg GAE/gG1 extract). Analysis was done 3 times, mean, standard deviation and standard error were calculated and applied.

Determination of total flavonoids (Aluminium chloride colorimetric method): Total flavonoid content was estimated quantitatively using aluminium chloride colorimetric assay following the protocol of Zhishen *et al.*[29]. The 1 mL extract was added in 2.8 mL of distilled H_2O followed by 0.1 mL solution of potassium acetate (prepared as 1 mg mL^{-1}) and 0.1 mL solution of 10% aluminium chloride ($AlCl_3$). Absorbance of this mixture was noted at 415 nm, after keeping the mixture standstill for 30 min. The flavonoid content was estimated as Quercetin equivalent, 10-100 μg mL^{-1} quercetins was taken as a standard and a calibration curve was plotted. Analysis was performed thrice.

Determination of alkaloids: Quantitatively alkaloids were estimated using the previously described method[25]. The 200 mL 10% Acetic acid (prepared in ethanol) was mixed with 5 g leaf extract. The solution was covered and kept to settle down for 4 hrs. After filtration, the filtrate was boiled in a water bath till to be about 1/4th of the initial volume, in the concentrate drops of conc. ammonium hydroxide (NH_4OH) was dribbled till the appearance of a precipitate. The precipitate formed was washed with dilute NH_4OH and finally filtered. After filtration, the residue was dried and then weighed. The amount of alkaloid present was calculated using the formula:

$$\text{Alkaloid (\%)} = \frac{\text{Final weight of the sample}}{\text{Initial weight of the extract}} \times 100$$

Determination of total antioxidant activity (Phosphomolybdenum method): The antioxidant capacity was assessed according to method of Prieto *et al.*[30] H_2SO_4 (0.6 M), sodium phosphate (28 mM), ammonium molybdate (4 mM) were mixed together to form a 3 mL solution, to this 0.3 mL extract was added. The solution was incubated in a water bath for 90 min, at a temperature of 95°C. Later solution cooled off and absorbance noted at 695 nm. Methanol (0.3 mL) without extract and reagent solution (3 mL) was used as blank. Using ascorbic acid as a standard reference calibration curve was derived from varied concentrations of ascorbic acid, ranging from 1000, 500, 250, 125, 62.5 to 31.25 μg mL^{-1}. Analysis was done 3 times.

Determination of antioxidant efficacy (DPPH radical scavenging activity): The DPPH radical scavenging effect was estimated by Liyanapathirana and Shahidi method[31]. A solution of DPPH (0.135 Mm solution) was prepared in methanol, 1 mL of which was mixed with 1.0 mL aqueous extract under different concentrations (varying from 0.2-1 mg mL^{-1}). After mixing, the reaction mixture was kept

for half an hour under dark conditions. Absorbance was recorded spectrophotometrically at 517 nm. DPPH scavenging capacity was estimated using the equation:

$$\text{DPPH scavenging activity (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

Where:

$Abs_{control}$ = DPPH and methanol absorbance

Abs_{sample} = DPPH radical+sample extract/standard absorbance

The IC_{50} value was calculated using AAT Bioquest IC_{50} calculator[32], lower values of IC_{50} indicates greater antioxidant capacity.

Determination of hydrogen peroxide radical scavenging capacity: The capacity of extracts to scavenge H_2O_2 was evaluated using Ruch *et al.* methodology[33]. The 40 mM Hydrogen peroxide solution was made in phosphate buffer (with pH 7.4). The 1 mL methanolic leaf extract (100 μg mL^{-1}) was mixed with H_2O_2 solution (0.6 mL), after waiting for 10 min mixture's absorbance was noted at 230 nm. Phosphate buffer alone acted as blank. Analysis was performed thrice and values were averaged. Percentage (%) scavenging by *A. dhufarensis* extract was estimated by the formula:

$$\text{Scavenged } (H_2O_2)\ (\%) = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

Evaluation of *in vitro* anti-inflammatory activity: Chandra *et al.*[34] protein denaturation method were applied to check the anti-inflammatory activity of extract. 2.8 mL solution of phosphate buffered saline (pH of 6.4) was mixed with 2 mL of extract (100, 200, 300, 400 and 500 μg mL^{-1}) to this 2 mL of egg albumin (from fresh hen's egg) was added. Mixture was incubated at 27±1°C for 15 min, thereafter heated in a water bath at 70°C for 10 min to induce denaturation, after cooling absorbance was noted at 660 nm, with double distilled water as blank. Acetyl salicylic acid under different conc. (100, 200, 300, 400 and 500 μg mL^{-1}) was used as standard reference. Test was performed thrice and values averaged. The % inhibition of protein denaturation was estimated as follows:

$$\text{Inhibition (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{test}}}{\text{Abs}_{\text{control}}} \times 100$$

Abs = Absorbance

Proteinase inhibitory activity: The Proteinase Inhibitors (PI) of *A. dhufarensis* were quantified[35]. The 2 mL of reaction mixture consisting of 1 mL of leaf extract under varied conc (100, 200, 300, 400 and 500 μg mL^{-1}), 0.06 mg trypsin, 1 mL Tris-HCl buffer (20 mM, pH 7.4) was kept for incubation at room temperature for 10 min. One mL Casein 0.8% (W/V) was added to this mixture and re-incubated for 20 min. To stop the reaction, 2 mL 70% perchloric acid was added at the end of incubation and mixture centrifuged at 7830 rpm for 15 min. Absorbance was read at 210 nm with Tris-HCl buffer solution as control, the experiment done in triplicate and mean calculated. The IC_{50} value was calculated using AAT Bioquest IC_{50} calculator.

RESULTS

The phytochemicals screening of the leaf extract depicted the presence of anthraquinones, saponins, alkaloids, glycosides, flavonoids, tannins, terpenoids, phytosterol, coumarins and quinones in Aloe*dhufarensis* leaf extracts as summarized in Table 1. Anthraquinones, alkaloids, coumarins, saponins and glycosides were present in strong intensity while flavonoids, terpenoids and Quinone in moderate intensity. Steroids were absent in the *A. dhufarensis* extract.

The total phenolic content depicted as Gallic acid equivalents/gram dry extract weight of the extract was determined from the regression equation of standard Gallic acid calibration curve and was recorded to be 452±3.2 mg GAEg^{-1} showed in Table 2. Flavonoid content was quantified as quercetin equivalents (QE g^{-1}) dry extract weight of extract, derived from the regression equation of the calibration curve of quercetin and the flavonoid in extracts of *A. dhufarensis* was recorded to be 44.16 mg of QE g^{-1} of dry extract. The total alkaloid content as was found to be 51.53±1.34 mg g^{-1}. As the total antioxidant capacity, is based on the reduction of Molybdenum (VI) to Mo(V) by the antioxidant compounds of sample extract, it produces green colored Phosphomolybdenum V complex and is depicted as Ascorbic Acid Equivalents (AAE). The TAC of *A. dhufarensis* extracts was recorded to be 256±1.4 mg AAE g^{-1} showed in Table 2.

DPPH radical scavenging activity: The *A. dhufarensis* leaf extract displayed strong DPPH scavenging activity proportional to the concentration. The scavenging activity was recorded to vary from 53-86.0% as the concentrations progressively increased from100-500 μg mL^{-1}, whereas DPPH scavenging activity of BHA and α-tocopherol at

Table 1: Different phytochemicals in methanolic extract of *Aloe dhufarensis*

Phytochemical screening	Exp.1	Exp. 2	Exp. 3	Mean
Test for Anthraquinones	++	++	++	++
Test for Saponins	++	++	++	++
Tests for Flavonoids	+	+	+	+
Tests for Tannins	+	+	+	+
Tests for Glycosides	++	++	++	++
Test for Terpenoids	+	+	+	+
Test for Steroids	-	-	-	-
Test for Alkaloids	++	++	++	++
Test for Phytosterol	+	+	+	+
Test for Coumarins	++	++	++	++
Test for Quinones	+	++	+	+

Table 2: Total phenolic content, total flavonoid content, total alkaloids and total antioxidant capacity in *A. dhufarensis*

Total phenolic content (mg GAEg^{-1} of dry mass)	Total flavonoid content (mg QE g^{-1} of dry mass)	Total alkaloids(mg g^{-1})	Total antioxidant capacity (mg AAE g^{-1})
452±5.2	44.16±1.82	51.53±2.34	256±3.4

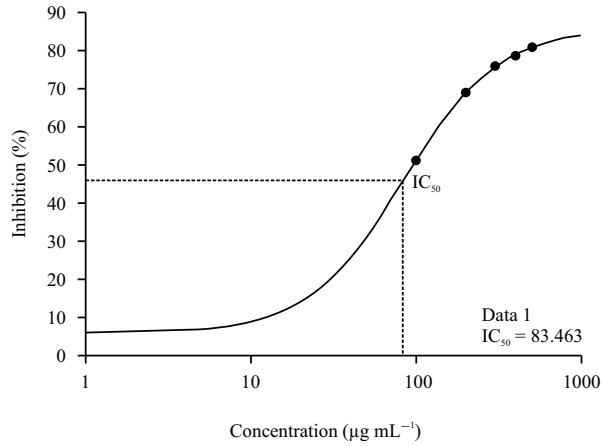

Fig. 1: Percentage scavenging and IC_{50} values for methanolic extracts of *A. dhufarensis* for DPPH radical scavenging

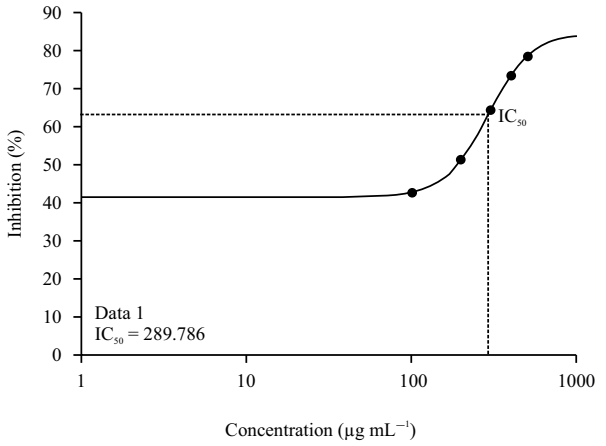

Fig. 2: Percentage scavenging and IC_{50} values for methanolic extracts of *A. dhufarensis* for Hydrogen peroxide scavenging activity

500 µg mL^{-1} was observed to be 76.32 and 82.56%, respectively. Using the AAT Bioquest IC_{50} calculator the dose-response curve was generated and the IC_{50} value calculated which was found to be 83.46 µg mL^{-1} showed in Fig. 1.

Hydrogen peroxide scavenging activity: Hydrogen peroxide (H_2O_2) scavenging by any plant sample is based on the electron-*donating* ability of plant phenolics to Hydrogen peroxide and thus neutralizing H_2O_2 to water. The *A. dhufarensis* leaf extracts exhibited strong hydrogen scavenging activity of 75.32% at 500 µg mL^{-1} concentrations, H_2O_2 scavenging activity of BHA was 78.6% and for α-tocopherol was 83.4%. The IC_{50} value of extract for Hydrogen peroxide scavenging was observed to be 289.786 µg mL^{-1} showed in Fig. 2.

Anti-inflammatory activity: All the different concentrations (100-500 µg mL^{-1}) of A. *dhufarensis* extracts displayed inhibitory effects on protein denaturation. As denaturation of proteins is the main cause of inflammation, anti-inflammatory activity of the *A. dhufarensis* leaf extract was evaluated by using both protein denaturation method and Proteinase inhibitory activity. The percentage inhibition of protein (albumin) denaturation was observed to be 89.95±1.83% in Fig. 3. From the results it is clear that A. *dhufarensis* effectively reduces of heat induced denaturation of albumin protein. The percentage inhibition by extract was as good as diclofenac sodium, the standard (having IC_{50} value 47.04 µg mL^{-1}) as IC_{50} of *A. dhufarensis* extracts for inhibition of protein denaturation was observed to be 50.407 µg mL^{-1} in Fig. 4. A. *dhufarensis* extracts exhibited substantial anti-proteinase activity, proteinase inhibitory activity ranged from 63.94% at 100 µg mL^{-1} to 76.53% at 500 µg mL^{-1}, with a IC_{50} value of 58.84 µg mL^{-1} (Fig. 4).

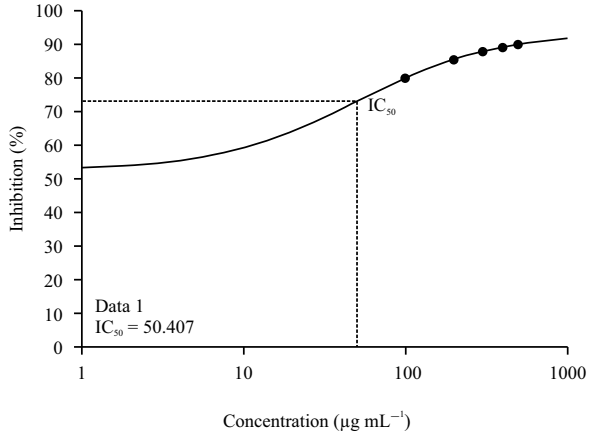

Fig. 3: Percentage inhibition of protein denaturation and IC_{50} values for methanolic extracts of *A. dhufarensis* for inhibition of protein denaturation

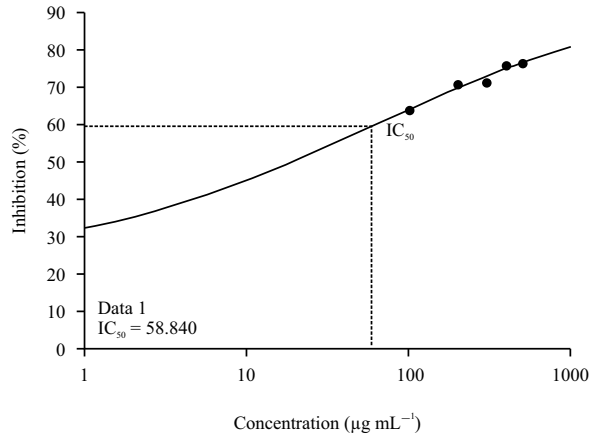

Fig. 4: Percentage proteinase inhibitory activity and IC_{50} values for methanolic extracts of *A. dhufarensis* for proteinase inhibitory activity

The Antimicrobial activity of methanolic *A. dhufarensis* leaf extracts of were estimated against 4 selected bacterial strains employing disc diffusion method and the activity was determined with regard to the zone of inhibition. The methanolic leaf extracts of *A. dhufarensis* exhibited considerable antibacterial capacity against all of the 4 tested bacteria showed in Table 3. Leaf extracts demonstrated highest antibacterial activity against the gram -ve *E. coli* with a Zone Of Inhibition (ZOI) of 13 mm, followed by *S. aureus*, *P aeruginosa* and least inhibition was observed for *B. subtilis* (7 mm).

Minimal inhibitory concentration observation: As minimum inhibitory concentration is the lowest concentration of any chemical/compound at which a given microbe couldn't display any noticeable growth after 24 hrs of incubation. All 4 bacterial strains showed visible inhibition. The MIC values for *E. coli* were the least at 250 µg mL^{-1} of the extract, *for S. aureus* and *P aeruginosa*, the MIC value was 500 µg mL^{-1} of the extract. Leaf extract was found to be least effective against *B. subtilis* (MIC 1000 µg mL^{-1}).

Table 3: Inhibition zone of methanolic extract in *A. dhufarensis* with different bacterial strains

Bacterial strain	Inhibition zone (mm)
Bacillus subtilis	7.0±0.272
Staphylococcus aureus	10.5±0.404
Escherichia coli	13.0±0.233
Pseudomonas aeruginosa	8.0±0.360

DISCUSSION

The present study was undertaken to validate the traditional use of *A. dhufarensis* in wound healing, to treat diabetes, headaches, fever, skin disorders, aching limbs and constipation as the pharmacological effects of this important species have not been fully explored.

An elevated oxidative stress because of abundant formation of reactive oxygen species and induced inflammation has been identified are the two major factors which play a definitive role in development and gradual advancement of various life-endangering ailments like Diabetes mellitus, neurodegenerative and wound infection[36]. Chronic or sporadic hyperglycaemia triggers various metabolic signalling pathways, inducing progressive inflammation in vessels and nerves, overproduction of reactive oxygen species, leading to oxidative stress, cytokines secretion and cell death consequently leading to diabetic complications[37,38].

Wound healing is a complex progressive mechanism involving a succession of overlapping events involving platelet initiated haemostasis[39,40], leading to coagulation under the influence of mediators and formation of fibrin clot. Further Platelet degranulation influences activation and infiltration of pro-inflammatory leukocytes, such as neutrophils, Monocytes and macrophages leading to the inflammatory phase. These leukocytes play role in the next proliferative phase. Macrophages and neutrophils produce many inflammatory mediators and cytokines like interleukin-1β, interleukin (IL)-6, tumor necrosis factor-α (TNF-α) and Nitric Oxide (NO)[41]. Under normal wound physiology after carrying various functions neutrophils undergo apoptosis triggering a progression out of the inflammatory phase. However in some cases neutrophils can generate excessive of NO and other unstable reactive oxygen radicals which damages cell

membrane, destroying ECM, in normal tissues around the wound and thus triggering extra generation of pro-inflammatory mediators like IL-1 and TNF-α leading to amplified inflammation and impaired wound healing[42]. The unstable reactive free radicals donates or abstracts electrons from DNA, proteins and fatty acids of the cell membranes, thus altering protein structure and their functionality, fragmenting the membranes and thus disrupting the proliferation of new cells at the site of healing. The inhibition of pro-inflammatory mediators like TNF-α may regulate the process of wound healing[43]. Oxygen radicals are toxic waste products which produce oxidative stress at the time of the inflammatory phase of wound healing. Overexposure to oxidative stress attributable to high levels of reactive oxygen species results in impaired wound healing[44]. Thus the radical scavenging agents and anti-inflammatory agents can be effective in wound healing process.

Several phytochemicals like phenols and flavonoids, alkaloids and triterpenes are important natural antioxidants capable of scavenging reactive free radicals, inhibiting oxidation and preventing cellular damages due to enhanced oxidative stress[45,46]. The phytochemical screening of leaves of *A. dhufarensis* extracts exhibited the presence *substantial* amount of anthraquinones, saponins, coumarins, alkaloids, flavonoids, phenols and tannins.

Anthraquinones found in latex and gel are well known for their laxatives and potent purgative effect due to their stimulation of mucus secretion and increased intestinal peristalsis[47] and are also known for powerful analgesic effect[48,49]. Presence of substantial amount of anthraquinones in *A. dhufarensis* authenticates its traditional use for constipation, headaches, fever and aching limbs.

Appreciably high amount of saponins, were observed in *A. dhufarensis*. Saponins exhibit substantial anti-inflammatory,[50] anti-diabetic, antibacterial[51] and antiseptic activity[52]. Saponins exert their potential anti-diabetic effect by rejuvenating insulin, resulting in lowered blood glucose level, elevated plasma insulin level, stimulating release of insulin (from pancreas) and by blocking glucose formation in the bloodstream[53]. Antibacterial potential of saponins is ascribed to their capability to cause excessive discharge of some enzymes and specific proteins from within the cell[54].

Plant polyphenols are also found to accelerate the wound healing process by exhibiting significant initially required pro-inflammatory activity, proliferation of fibroblasts, formation of new blood vessels and amplification of new dermal cells[55].

Important flavonoids like catechin, luteolin, rutin and apigenin are established for their wound healing properties[56] through inhibition of excessive fibroblast growth, increased collagen synthesis, improving cross-linking of collagen and inhibiting the breakdown of soluble collagen[57]. Flavonoids along with saponins by inhibiting the enzymes involved in pro-inflammatory mediators proliferation are known to play significant part in wound healing and health promotion[58,59]. Flavonoids are widely recognized for their antibacterial, diuretic, laxative antispasmodic and cytotoxic effects. Phenolic acids along with alkaloids exhibit effective analgesic properties[60].

Polyphenols are the major antioxidant compounds found in plants, attributing their antioxidant potential to their redox properties. Antioxidant are substances that inhibits the accumulation of free radicals like hydroxyl radicals (OH-), superoxide radical anion ($O_2^{\bullet -}$) and other reactive oxygen H_2O_2 and nitrogen species like NO and N_2O_3, respectively[61] These ROS at elevated concentrations generate oxidative stress resulting in various detrimental effects, like activation or deactivation of enzymes, altering membrane permeability due to lipid peroxidation, nucleic acids and altered lipid-protein interaction[62,63]. Multiple OH group of phenolic compounds especially phenolic acids, imparts strong anti-oxidant potential to plant phenolics by neutralizing the free radicals like $O_2^{\bullet -}$ and HO$^{\bullet}$ and scavenging other ROS[64]. Other type of phenolic compounds for example flavonoids, exert their anti-oxidant potential by activating antioxidants enzymes, inhibit oxidases especially nitric oxide and peroxidase, deescalate α-tocopherol radicals and chelate metal catalyst[65]. According to the present study, phenolic compounds are the present in substantial amount in *A. dhufarensis* and are capable of donating hydrogen atom to the ROS thus contributing to the pharmacological effects associated with this species.

Results verify that *A. dhufarensis* leaf methanolic extracts have proton-donating abilities and potent antioxidant ability as they stabilize the free radical of DPPH to and reducing it to diphenylpicryl hydrazine. Similar findings were observed[3] in *A. dhufarensis*. IC_{50} value is negatively correlated with the antioxidant activity, the less is the IC_{50} value of extract, the greater is its antioxidant potential.

Within cells H_2O_2 is generated during the reduction of O_2 to water via one-electron successive pathway. Where molecular oxygen is first changed into $O_2^{\bullet -}$ (superoxide anion radical) and this radical accepts one electron and two protons, yields hydrogen peroxide (H_2O_2).

Hydrogen peroxide, is an unstable and a reactive oxygen species with an important property to freely cross cell membranes and by accepting one electron dissociate into hydroxyl radical (HO$^{\bullet}$) and (OH^-) ion[66]. Most of the detrimental effects attributed to hydrogen peroxide like lipid peroxidation in biological membranes, disrupting protein structure by

reduction of disulphide bonds are mediated via the hydroxyl radical[67]. Antioxidant capability of a plant extract is ascribed to their phenolics which by donating electron can reduce hydroxyl radical to H_2O. Low IC_{50} value of extract in the present study indicates a strong hydroxyl radical scavenging capacity of *A. dhufarensis* phenolics.

As discussed above Inflammation and escalated oxidative stress is associated with a number of life-long diseases. Inflammation, a critical natural counter action of body immune system to injury or stress occurs via creation of certain pro-inflammatory cytokines such as IL-1β, interleukin-6 and TNF-α and their interaction with their specific receptors like interleukin-1 receptor and tumor necrosis factor receptor[41]. Denaturation of proteins is one of the prime attribute leading to inflammation and the inhibition of protein denaturation may help in averting inflammatory conditions[68]. Proteases are catalytic enzymes hydrolysing peptide bonds of proteins and are pivotal in inflammation as the leukocytes proteinase carries out tissue damage during an inflammatory response. Inhibition of protease activity will prevent inflammation.[69] Considerable inhibition of protein denaturation and of protease activity by *A. dhufarensis* leaf extract confers considerable anti-inflammatory activity to the extract.

Phytochemical like tannins via inhibition of inflammatory mediators like cytokines and COX-2[70], Saponins by inhibiting nitric oxide (NO) by down regulation of lipopolysaccharide induced iNOS expression[50], phenolic acids by deterring TNF-α expression[71], Flavonoids and coumarins by halting cyclooxygenase and the lipoxygenase pathways of arachidonic acid metabolism[72] are effective anti-inflammatory agents. Strong presence of these phytochemicals imparts potent anti-inflammatory potential to *A. dhufarensis*. Similar results were obtained[73,74] in Aloe ferox and in *Aloe vera*.

Good antimicrobial activity of *A. dhufarensis* against all 4 bacterial strains can also be ascribed to the existence of saponins, alkaloids, tannins, flavonoids and terpenoids. Saponins and terpenoids exhibit their antimicrobial effect by impairing bacterial cell membranes[75,76], Alkaloids by increasing membrane permeability through protein denaturation[77] and flavonoids by suppressing nucleic acid synthesis and effecting the membrane permeability[78]. Many previous studies on various species of genus Aloe has validated their antimicrobial activity[79-81].

CONCLUSION

This work reinforces the scientific basis for the established traditional use of *A. dhufarensis* as an important healing herb as the results depicts strong radical scavenging capacity, potent anti-inflammatory and also antibacterial potential of leaf extracts of *A. dhufarensis* due to presence of substantial amount of anthraquinones, saponins, phenolic acids, alkaloids and flavonoids. The encouraging findings emphasizes on the possibility of the mainstream commercial exploration of the pharmaceutical potential of *A. dhufarensis* in parallel to other commercial species of the Aloe genus.

SIGNIFICANCE STATEMENT

This study discovered the untapped pharmaceutical and pharmacological potential of an endemic medicinal herb that can be commercially explored on national and international scale. This study will help the researchers to uncover the critical areas of plant stress physiology under specialized realms of microenvironment and adaptive response via elevated generation of secondary metabolites.

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