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Comparative *in vitro* anti-oxidant and anti-fungal potential profiles from methanol extract of *Fagonia indica, Fagonia bruguieri* and *Fagonia paulayana*

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Abstract
Plants have proved to be the cure of many harmful diseases therefore, the demand of herbal medicines is increasing day by day. According to the previous research data *Fagonia*, a genus of 45 species is identified as a very potential medicinal plant against multiple diseases but poorly identified in Pakistan. As most of the biological research depends on the species identification and authentication, the sustainable method for the identification is now accepted as a molecular based method that employ DNA sequences as taxon 'barcodes' because taxonomic expertise are not serving the sole purpose. Therefore, we collect various *Fagonia* samples from different localities of Pakistan. Out of 12 samples, 7 were identified as *F. indica*, 3 as *F. paulayana* and 2 as *F. bruguieri*, and the potent species we have here in Pakistan is *F. indica* not *F. cretica* as they both are least similar. After that, the anti-oxidant, free radical scavenging and anti-fungal activities of methanol extracts of identified *Fagonia* species were checked. The best results were shown by *F. indica* then *F. bruguieri* and least were shown by *F. paulayana*.

Keywords: Anti-oxidant, Anti-fungal, *Fagonia indica*, *Fagonia bruguieri*, *Fagonia paulayana*

Introduction
From the beginning of the human civilization, there is a quest for finding novel, cheaper and effective cure of a disease. Consequently human beings have explored natural resources of the planet (Khan *et al.*, 2015) [12]. The world is familiar with thousands of medicinal plants since time immemorial and even today, 80% of the entire world are dependent on the traditional medicines. Present day antibiotics are becoming rapidly compromised because of antibiotic resistance which has diverted attention towards alternative solutions like plant based medicines. In Pakistan, folklore medicines has deep foundations in the society. The most practiced traditional systems in Pakistan include Greek medicines, Greeco-Islamic and Prophetic medicines, Chinese medicines, and Ayurveda. (Khalil *et al.*, 2014) [13].

Pakistan is a blessed region having a number of important medicinal plant species. Certain members of Zygophyllaceae such as *Fagonia* (Beier, 2005) [5], a morphologically cryptic genus is well known for its therapeutic potential. Mostly *Fagonia* species are represented as woody herbs andthey are represented by 45 species distributed in deserts and dry zones of the world. It naturally grows in warm areas and in dry calcareous rocks throughout the Mediterranean regions of Africa to the deserts of central Asia (Rizvi *et al.* , 1996) [21]. Recent research indicated that genus *Fagonia* can be an important target for the discovery of valuable drugs or relevant chemical entities. Phytochemical studies showed that the genus has a wide array of important chemicals likes aponins, flavonoids, terpenoids, and sterols etc that can be of therapeutic value (Alam *et al*., 2011) [2, 3]. *Fagonia* is already reported for its use in hepatic, neurological and haematological problems (Aggarwal, *et al.*, 2011) [1]. Aqueous extracts of *Fagonia* are used to treat early stage cancers (Saeed, 1969) [22] while the paste is applied externally on tumours and swellings and it is also a good source of iron, magnesium, and zinc (Shad *et al.*, 2002) [25]. *Fagonia* is also reported to be diuretic, anti-haemorrhagic, antipyretic, anti-dysenteric, anti-hepatotoxic and also a stimulant (Anil, *et al.*, 2012) [3]. Some other studies indicate the antimicrobial, anti-fungal, anti-cancer and anti-oxidant properties of *Fagonia* (Sajid, *et al.*, 2011; Lam, *et al.* , 2012; Rawal, *et al.*, 2009) [23, 15, 20]. Traditionally it is also used for asthma, toothache, kidney diseases and urinary discharge (Baquar, 1989) [4].

However, *Fagonia* species, shows significant morphological and anatomical similarity making their taxonomic identifications ambiguous. Recently *Fagonia cretica* was reported for being highly active against breast cancer but later on the specie was identified as *Fagonia indica* or its sister specie *Fagonia paulayana*. Later on Matt *et al.*(2014) [17] proposed the specie as *Fagoniaindica* and not *Fagonia paulayana*based on molecular studies. He further proposed that *Fagonia cretica* was not present in Pakistan (Matt *et al.*, 2014) [17].

Traditional taxonomic identifications are on based on organoleptic method, micro/macroscopic characters and chemical profiling but the aforementioned methods were unable to discriminate *Fagonia* species from one another (Kumar *et al.*, 2009) [29]. With the increasing trade of herbal products, herb substitution, mixing, misidentifications and adulterations has become a routine (Newmaster *et al.*, 2013)

[18]. Therefore DNA based standardization of plant material has become a necessity. To overcome such problems, (Hebert *et al*., 2003a) [10] purposed a successful molecular and computational based method of DNA-barcoding to identify existing species as well as to discover new species. DNA-based methods are more authentic and reliable because DNA is stable, available in every tissue and above all, it is unaffected by external factors (Sucher and Carles, 2008) [27]. Such DNA based methods are very useful to identify and authenticate medicinal plants from which a number of medicines originates. The use of DNA barcodes parallel to the conventional methods will promote the authenticity of natural medicines. DNA-barcoding is an integrated taxonomic system where the regions of DNA consists of <1000 bp, are used and termed as DNA-barcodes (Li *et al.*, 2011b) [16]. In the present research, DNA barcoding was used *(matK, rbcL, trnH-psbA*) for identification of fagonia species across different regions of Pakistan. The sequences generated in the study were used along with the retrieved sequences from GenBank to perform a basic phylogenetic analysis. The selected plants were also subjected to a basic in vitro biological assays using the crude methanolic extracts.

Materials and Methods

Collection of plant material

*Fagonia*spp were collected from different areas of Pakistan (table 2) and identified by Dr. Mushtaq Ahmad (Department of Plant Sciences) and Prof. Dr. Abdul Rasheed (Department of Botany, University of Peshawar). Herbarium specimens were deposited in the herbarium of Molecular Systematics and Applied Ethnobotany Lab at the Department of Biotechnology Quaid-i-Azam University.

DNA-barcoding protocol

DNA extraction from 0.3g of each sample was done by 2% CTAB method, genomic DNA then confirmed by 1% agarose gel electrophoresis, and visualized under UV light by Dolphin doc plus gel documentation system (wealtec). The PCR reactions for the regions of *rbcL, matK,* and*psbA-trnH*was carried out in PEQSTAR PCR using KAPA3G plant PCR kit (Schori *et al.*, 2013) [7]. PCR products were then confirmed by 1% agarose gel electrophoresis. The PCR clean-spin protocol) by using AxyPrep PCR clean up kit was used for the purification of PCR product. The purified product was sequenced in USA at Ohio University's Genomic Facility and analyzed using ABI, 3130xl Genetic Analyser (Applied Biosystems Carlsbad, California, and USA). Contigs and consensus sequences were produced using Geneious 6.1.6 (Biomatters Ltd., Auckland, New Zealand).

Extraction and stock dilutions

Shade dried samples were grounded to fine powder, comprised of leaves, stems, spines, fruits and flowers collectively. Powdered plant material were then soaked in methanol used as a solvent in the quantity of (30gm/90ml) for 24-48 hours then sonicated to rupture plant cells. Solution was then filtered using cloth. The residue obtained were again soaked in respective solvent following the same period and filtered again. All the filtrates were evaporated at room temperature to obtain crude extracts. Sample stock dilutions were prepared in methanol as 4 mg/ml Methanol.

Antioxidant Assays

Following five antioxidant activities were carried out to measure the efficiency of crude methanol extract of *Fagonia* spp.

DPPH radical scavenging assay

The DPPH, was prepared by dissolving 9.6 mg reagent/100 ml methanol. Plant extracts (4 mg/ml methanol) were taken in the quantity of 10, 5, 2.5 and 1µl respectively, the quantity of DPPH added to samples was 190, 195, 197.5 and 199 µl. 20 µl of methanol used as a negative control with 180 µl of DPPH reagent were mixed to it. 4 mg/ml stock solution of Ascorbic acid (Mol. Wt:176.12 g/mol) in methanol were used as a positive control in the quantity of 20, 8, 4, 2 and 1 µl and the amount of DPPH added to it were 180, 192, 196, 198 and 199 µl respectively. Incubation was done for 1 hour at room temperature, absorbance readings were noted at 517 nm wavelength on microplate reader (ELx 800 BioTek). The percent IC_{50} values of extracts were calculated as Ascorbic Acid Equivalent (AAE) µg/mg of extracts (Chew *et al.*, 2009)

Total antioxidant capacity (phosphomolybdenum method)

About 20 µl of plant extracts (4 mg/ml methanol) were transferred to the eppendorf tubes with 180 µl of phosphomolybdenum reagent and incubated in water bath at 95 ºC for 90 minutes. After incubation eppendorf tubes were cooled at room temperature. Then 20 µl of methanol and 20 µl of 4mg/ml Ascorbic acid were used as negative and positive control respectively while adding reagent in the same quantity as those were in samples. Absorbance was taken at 630 nm wavelength on microplate reader (ELx 800 BioTek) (Prieto *et al.*, 1999) [19]

Reducing power

About 40 µl of plant extracts (4 mg/ml methanol) taken in eppendorf tubes, 50 µl of 1% Potassium ferricyanide and 50 µl of 0.2M Phosphate buffer having pH 6.6 were added to the samples. Incubation was done at 50 ºC for 20 minutes. After incubation 50 µl of 10% Trichloroacetic acid added to the mixture and centrifuged at 3000 rpm for 10 minutes. After centrifugation process 166.66 µl of upper layer of centrifuged mixture were taken in 96 well microplate and 33.3 µl of 0.1% ferric chloride were mixed to the isolates. As a negative and positive control Methanol and Ascorbic acid (1 mg/ml ascorbic acid) were used respectively. Readings taken at 630 nm wavelength on microplate reader (ELx 800 BioTek) (Ferreira *et al.*, 2007) [8]

Total flavonoid contents by using aluminium chloride ($AlCl_3$)

About 20 µl of plant extracts (4 mg/ml methanol) were added to 96 well microplate, to make a total reaction volume of 200 µl, 10 µl of 10% aluminum chloride, 10 µl of 1M potassium acetate and 160 µl of distilled water were added to plant material. As a negative control, 20 µl of Methanol were used and 4 mg/ml quercetin in methanol were used as a positive control with a final concentration of 50, 25, 20, 10, 5 and 2.5 µg/ml (10, 5, 4, 2, 1 and 0.5 µl), distilled water quantity added to quercetin was 170, 175, 176, 178, 179 and 179.5 µl respectively. Methanol and quercetin were used as a standred. Readings were taken for further analysis at 415 nm wavelength on microplate reader (ELx 800 BioTek) (Haq *et al.*, 2012) [9]

Total phenolic content by using Folin- Ciocalteu

About 20 µl of plant extracts (4 mg/ml methanol) were poured in 96 well microplate mixed with 90 µl of Folin-Ciocalteu reagent. Mixture was incubated at room temperature for 5 minutes. After incubation 90 µl of sodium carbonate added to it, mixed well and incubated again at room temperature for 90 minutes. methanol were used as a negative control and 4mg/ml Gallic acid in methanol as a positive control in the final concentration of 25, 20, 15, 10 and 5 µg/ml (5, 4, 3, 2, 1 µl). Amount of methanol added to Gallic acid was 15, 16, 17, 18 and 19 µl respectively Readings were taken at 630 nm wavelength on microplate reader (ELx 800 BioTek) a processed for further analysis (Singleton and Rossi., 1965) [26]

Antifungal activity

Filter paper discs (6mm) were dipped in (20 mg/ml methanol) plant samples by using sterile forceps and placed at proper positions on the medium. Antibiotic Clotrimazole (4 mg/ml) was used as control, plates were placed for incubation for 48-72 hours at 30 ºC and zones of inhibition were observed.

Results

In the present research, we have conducted separate molecular phylogenetic studies based on *matK, rbcL and trnh-psbA.* Phylogenetic trees were constructed using NJ (Neighbor Joining) method using software Geneious 6.1.6. The accessions downloaded and generated in the study are shown in Table 3. Our *rbcL*, *matK* and *trnh-psbA* results indicated that out of 12 species of *Fagonia* collected from different regions, 7 were found to be *Fagonia indica. 3* species as *Fagonia paulayana* and 2 as *Fagonia brugueri.* Phylogenetic tree based on *matK* gene region shows interesting relationship of *Fagonia Arabica* with all of the 12 species of *Fagonia* used in the study. It can be inferred that *Fagonia* species in the Indo-Pak region have evolved from *Fagonia Arabica* which is also supported by strong bootstrap score (100). Furthermore the clustering patterns are similar for *Fagonia paulayana* (Indian region), which is clustered together with Pakistani species but is placed separately in *matK* based phylogenetic tree with a low bootstrap score (59.5) as shown in Fig 1, 2 and 3. Our *rbcL* phylogenetic analysis indicates two main clusters that comprises. One comprises of *F. brugueri* and the rest comprises of *F. indica* and *F. paulayana.* All the *Fagonia indica* as well as *Fagonia paulayana* species are grouped together (Fig *rbcL*). *Fagonia acerosa* shows a possible ancestral relationship with *Fagonia brugueri* supported by bootstrap score (70.5). Figure 1, 2 and 3 shows the phylogenetic tree based on gene regions *rbcL, matK* and *trnh-psba* respectively.

Antioxidant Assay

DPPH Assay

The lowest IC_{50} value was seen for *Fagonia indica* (Table 1*)* which entails that this plant has strong free radical scavenging potential the remaining two plants the next one comes *Fagonia bruguieri* followed by *Fagonia Paulayana.*

Table 1. IC_{50} values of extracted plants concentrations

Plant specie	Extracts concentrations			IC_{50} (µM)
	100ug/ml	33ug/ml	11ug/ml	
Fagoniabruguieri	63	51	33	32.733
FagoniaIndica	78	58	41	24.16
FagoniaPaulayana	66	43	29	41.4

Total anti-oxidant assay

In our results the best TAO value is shown by *F. indica* (156 µg AA equivalent/mg methanol extract), while *F. bruguieri* and *F. paulayana* representing 130 and 120 µg AA equivalent/mg methanol extract respectively (Figure 1)

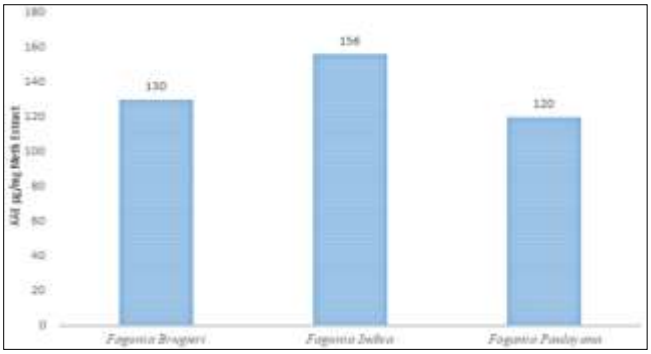

Fig 1: Denoted Total Anti-oxidant Power of *Fagonia* plants AAE= Ascorbic Acid Equivalent

Total Reducing power assay (TRP)

In our current study the best TRP value was noted for *F. indica* (173 µg AA equivalent/mg methanol extract), the second one comes *F. bruguieri* (141 µg AA equivalent/mg methanol extract) while the *F. paulayana* reveals the lowest value (113 µg AA equivalent/mg methanol extract) (Figure 2)

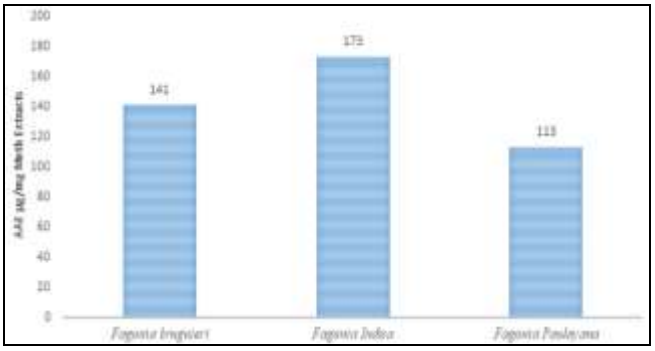

Fig 2: Total Reducing Power of three *Fagonia* plants

Total flavonoid contents

Like TPC the best flavonoid contents was shown by *F. indica* (192 µg QE equivalents /mg meth extracts), the second one comes *F. bruguieri* (188 µg QE equivalents /mg meth extracts) and lowest TFC was noted in *F. paulayana* (148 µg QE equivalents /mg meth extracts) (Figure 3)

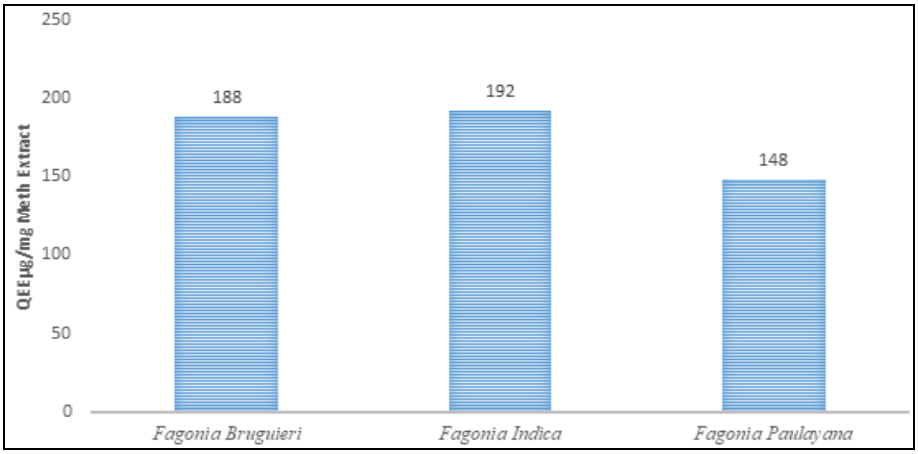

Fig 3: represents Total Flavonoid contents of three *Fagonia*plants QEE= Qurcetien Equivalent

Total phenolic contents (TPC)

The methanol extract of all three species of *Fagonia* were subjected to TPC, the best result was revealed by *F. indica* (187 µg GA equivalent/mg methanol extract) followed by *F. bruguieri* (158 µg GA equivalent/mg methanol extract) while the *F. paulayana* shows the lowest value among these three plants (121 µg GA equivalent/mg methanol extract) (Figure 4). The antifungal results are listed in Table 2.

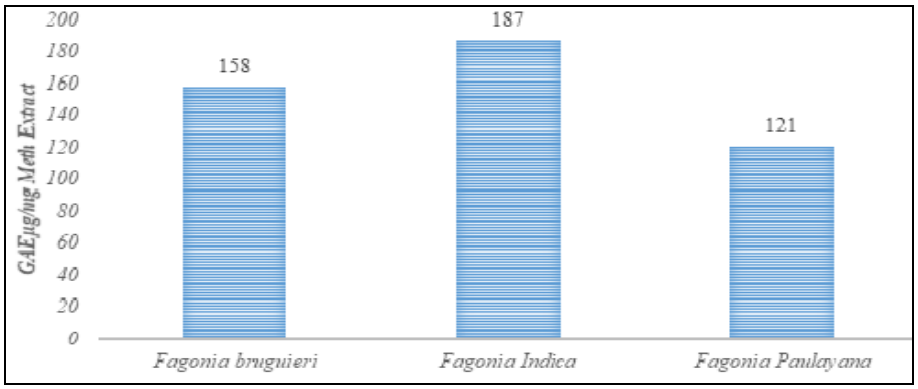

Fig 4: shows the Total phenolic contents of three *Fagonia* species, GAE =Gallic Acid Equivalent

Table 2: indicates no zone, + indicates small zones < 10 mm, ++ indicates medium zones > 10 mm, +++ indicates larger zones 15-20 mm & above

***Fagonia* species**	***Aspergillus niger***	***Aspergillus Fumigatus***	***Aspergillus Flavus***	***Mucor mycosis***
F. bruguieri	+++	+++	++	+++
F. Indica	++	+++	+++	++
F. Paulayana	++	++	++	+++

Table 3: Accession numbers of the sequences taken from Gene Bank

Accession numbers	**Description**
Y15028.1	*Tribulus macropterus (rbcL* gene)
AJ 133855.1	*Fagonia cretica (rbcL* gene)
KP259306.1	*Fagonia californica (rbcL* gene)
KP259297.1	*Fagonia acerosa (rbcL* gene)
KP259302.1	*Fagonia glutinosa (rbcL* gene)
AJ133856.1	*Fagonia luntil (rbcL* gene)
KP259307.1	*Fagonia paulayana (rbcL* gene)
KC593453.1	*Tribulus terrestris (psbA-trnH* gene)
HQ386692.1	*Tribulus lanuginosus (psbA-trnH* gene)
HG963626.1	*Guaiacum sanctum (psbA-trnH* gene)
KJ426753.1	*Guaiacum officinale (psbA-trnH* gene)
GU135354.2	*Tribulus cistoides (psbA-trnH* gene)
KF224985.1	*Tribulus terrertris (matK* gene)
GU135025.1	*Tribulus cistoides (matK* gene)
KR735139.1	*Balanites aegyptiacus (matK* gene)
EU002172.1	*Bulnesia arborea (matK* gene)
KM276890.1	*Fagonia Arabica (matK* gene)

Table 4: *Fagonia* Species collection areas

Test Samples	Codes Given	Location of Collection	Geographical Coordinates
Fagonia bruguieri	*Fagonia bruguieri* (N)	Nushki Balochistan	29.9333° N, 66.0167° E
Fagonia bruguieri	*Fagonia bruguieri* (M)	Mach, Bolan District, Balochistan	29.8667° N, 67.3333° E
Fagonia indica	*Fagonia indica* (HT)	Hattar, Haripur district, Kp	33.8503° N, 72.8522° E
Fagonia indica	*Fagonia indica* (K)	Khushal Ghar Bridge, Kohat	33° 0' 7" N, 71° 55' 40"E
Fagonia indica	*Fagonia indica* (B)	Burhan, Attock District, Punjab	33.8161° N, 72.5831° E
Fagonia indica	*Fagonia indica* (HB)	Hayatabad, Peshawar	33.9861° N, 71.4569° E
Fagonia indica	*Fagonia indica* (MZ)	Mulla Zai, Peshawar	32.413489°N,70.465826°E
Fagonia indica	*Fagonia indica* (A)	America, Oregon State University	44.5646° N, 123.2757° W
Fagonia indica	*Fagonia indica* (HZ)	Hazro, Attock district, Punjab	33.9097° N, 72.4928° E
Fagonia paulayana	*Fagonia paulayana* (RP)	Rawalpindi (Hakeem)	33.6000° N, 73.0333° E
Fagonia paulayana	*Fagonia paulayana* (I)	India	21.0000° N, 78.0000° E
Fagonia paulayana	*Fagonia paulayana* (L)	Lahore (Akbari mandi)	31.5497° N, 74.3436° E

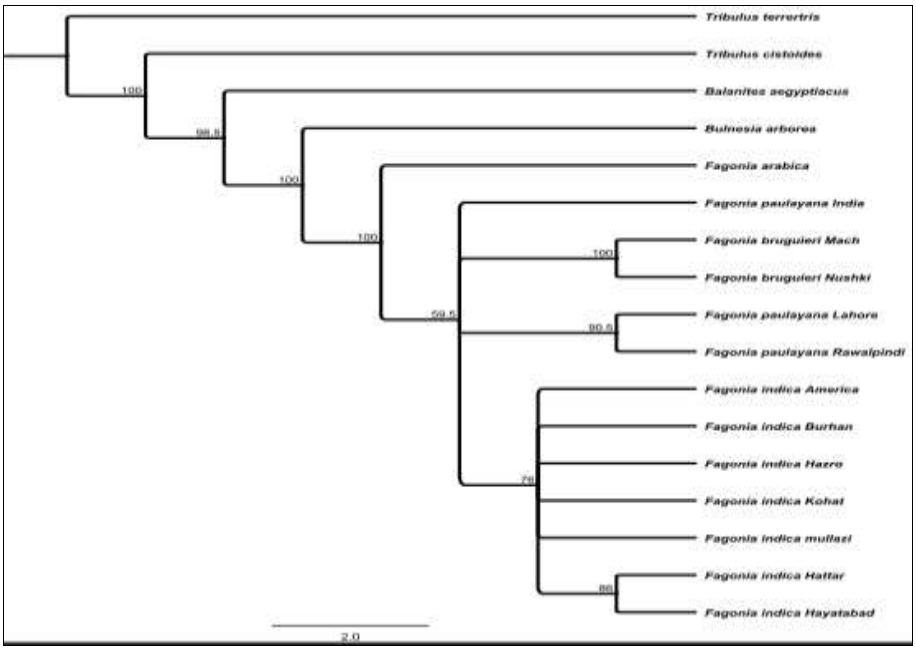

Fig 5: MATk Tree

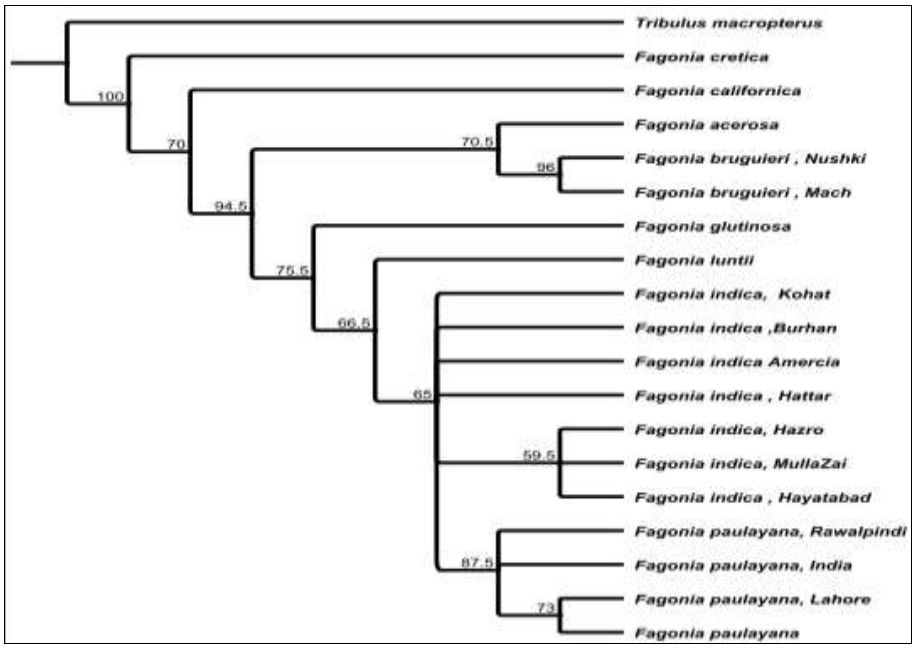

Fig 6: rbcl

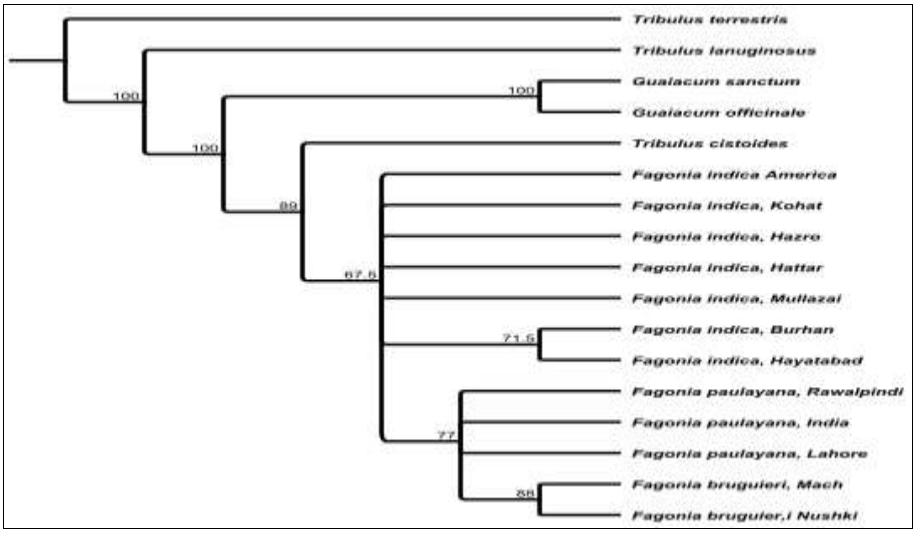

Fig 7: TRNHPSBA

Discussion

Misidentification due to the morphologically cryptic species has been the cause off substandard herbal products that leads to the loss of consumer confidence because of less efficacy. It is imperative to standardize the plant material on DNA level to ensure the quality of herbal product.Ravindra *et al*.(2012) reported the identification of *F. Arabica* (Batch number: C/6473) difficult because it did not matched any identification criteria. Likewise, (Ahsan *et al.*, 2006) claimed their plant as *Fagonia cretica* by giving collection location as "Sarwar Shaheed Chowk, District Thu'' without mentioning any method of authentication either. Resolving the taxonomic identification of *Fagonia* genus is a highly debated topic in scientific literature mainly because of its remarkable inters species resemblance.

Keeping this issue in mind, we have collected different *Fagonia* samples from multiple regions of Pakistan as shown in (Table 4). Our methodology included both the morphological study and DNA barcoding of three gene regions (*rbcL*, *matK*, and *psbA-trnH*) of these plants. By using Beier's revision of *Fagonia* as a reference we found out that both of our Baluchistan samples had thicker ridges on the angles as compared to the other samples which have evenly sized ridges. When checked under a stereomicroscope, both the Baluchistan samples had sepals while the other samples did not (Beier, 2005) [5] described that *F. bruguieri* has thicker ridges on the angles of the stem and thinner ridges in between and in *F. indica* they are of same size. So with two characters - uneven ridges and persistent sepals - in both Baluchistan samples and even ridges with deciduous sepals in other samples, we identified our Baluchistan sample as *F. bruguieri* and others are as *F. indica* or *F. paulayana*. As it is not easy to distinguish *F. indica* and *F. paulayana* as they have highly similar morphology except one ambiguous character that is the persistence of sepals (Beier, 2005) [5] but, they differ consistently in their sequence data for *rbcL*, *matK*, and *psbA-trnH* (Fig. 4-6). So it is easy to tell them apart by sequence data. Furthermore, *F. paulayana* does not have persistent sepals so we conclude some of our samples as *F. indica* and some as *F. paulayana.*

Further identification was done by the molecular analysis of these species by their three gene regions. When matched with the sequences of Kew taken as reference sequences we found out the correct identification of our samples as shown in table 1. Kew sequences included *Fagonia acerosa rbcL* (KP259297), *F. bruguieri rbcL* (KP259308, KP259304, KP259301), *F. cretica rbcL* (KP259298), and *F. paulayana rbcL* (KP259299, KP259307).The *rbcL* sequences of our samples have been assigned GenBank numbers as (KP259297-KP259308).

Our *rbcL* results suggest that *F. bruguieri* (N) is more similar to*F. acerosa* and *F. paulayana* (L) as compared to *F. indica* (HT) while least similar to *F. cretica*. On the other hand the sequence of *F. indica* (HT) showed maximum similarity with *F. paulayana* (L) then with *F. bruguieri* (N), and showed least similarity with the *F. cretica*. Then *F. paulayana* (L), then showed maximum similarity with its sister species *F. indica* (HT) then with *F. bruguieri* (N) and least similarity is observed with the GenBank sequence of *F. cretica*. These results clearly show that by *rbcL* gene analysis *F. cretica* is lest similar with all our three identified species. According to the results of *psbA-trnH*, we conclude the same pattern of similarities as concluded for *rbcL* gene analysis except for *F. cretica* as we do not have any GenBank data available for *psbA-trnH* gene of *F. cretica*. Molecular analysis of our third gene *matK* suggests that *F. indica* (HT) is more similar with *F. paulayana* (I) then with *F. arabica*and least similar with *F. bruguieri* (M). On the other hand, *F. paulayana* (I) is more similar with the *F. Arabica* GenBank sequence, then with *F. indica* (HT) and least with *F. bruguieri* (M). Again, *F. bruguieri* proved to be more similar with*F. Arabica* GenBank sequence and *F. paulayana* (I) then, with *F. indica* (HT).

DPPH is radical, very stable and purple coloured, readily scavenged by antioxidants under normal conditions. The purple colour of DPPH free radical turns yellow when an electron or hydrogen atom is donated by antioxidants. More intense colour means more scavenging potential of antioxidant sample. This test is used to measure oxidative damage done by free radicals. The lowest IC_{50} value was seen for *Fagonia indica*, IC50 (24.16) which entails that this plant has strong free radical scavenging potential than the remaining two species. The total anti-oxidant works upon the same principals as of DPPH but having different reaction kinetics and reducing substrates *in-vitro* however, physiologically these both have same mechanism of actions (Ignat *et al.*, 2011) [28]. In our results the best TAO value is

shown by *Fagonia indica* with a value of 156 µg AA equivalent/mg methanol extract.

The total reducing power of biological extracts depends on the presence of reductones that provide the hydrogen atoms to cleave the radical chain reaction (Walia *et al.*, 2009) [29]. Samples having more antioxidant potential reduce potassium ferricyanide (Fe3+) to potassium ferro-cyanide (Fe^{2+}) that converts into ferric ferrous complexes in the end. Then the complexes of ferric ferrous from the reaction mixture is analysed at 700nm. Again the best result for total reducing power was shown by *Fagonia indica*with a value of 173µg AA equivalent/mg methanol extract.

The total phenolic content of the methanol extracts of *Fagonia* species were measured by using Folin-Ciocalteu's reagent a mixture of phosphotungstate and phosphomolybdate. When the oxides of tungsten and molybdenum reduced, solution turns blue in colour, and monitored spectrometrically at 630 nm. In this investigation, methanol extract of *F. indica* showed up the highest phenolic content of 187 µg GA equivalent/mg methanol extraction. In total flavonoid, on the basic skeleton of flavonoid rings, various types of hydroxyl groups are present that scavenge free radicals. The position of hydroxyl groups is responsible for the stability of phenoxyl radicals created during the process of antioxidant action. Like TPC the best flavonoid content was shown by *Fagonia indica* with the value of 192 µg QE equivalents /mg meth extracts. Overall, the best results for both TPC and TFC were shown by methanol extract of *Fagonia indica*. Inappropriate and consistent use of fungicides cause resistance in fungal strains. Human health is on serious threat because of fungal resistance against antifungal drugs. Rise in population also cause resistance in microbes (Barret, 2002). Novel drugs production that have high effective antifungal activities with long term safety are the need of time to over-come the present scenario, if we really want to tackle the pathogenic fungal infections. Antifungal activities of three different *Fagonia species* showed positive results against all four fungal strains, some *Fagonia species* gave medium zones >10mm and some gave lager zones >15-20mm or above, against all four fungal strains.

Conclusion

In conclusion, on the basis of DNA sequence data generated in our research, it is indicated that *F. cretica* species is not available in Pakistan. The species assumed to be *F. cretica*is validated as *F. indica*. The values of TPC, TFC, scavenging of DPPH, total antioxidant and, total reducing power results have indicated that *Fagonia indica* has high antioxidant and radical scavenging potential as compared to *Fagonia bruguieri* and *Fagonia Paulayana.* As all three market samples of ours are identified as *F. paulayana* that showed least values of antoxidant potential and is directly hitting the herbal market's inauthentic and unreliable ways of trading as well as treatments of various diseases. While all these three species gave positive results against all four fungal strains and the methanol extracts of these three *Fagonia* spp could be explored for further *invitro* analysis.

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