Genetic profiling of *Cynodondactylon*speciesusing ISSR markers and its pharmacological activities

ABSTRACT

Aim:*Cynodondactylon*is a grass variety of the tropical and sub-tropical region belonging to the family Poaceae. In this study genetic profiling of 3 different varieties of *Cynodon dactylon* were done using ISSR primers.

Methodology: Polymorphism patterns were observed by amplifying DNA with 8 sets of primers such as OPA16, OPB11, OPC06 etc. PyElph tool was used to understand the genetic similarity of the samples and accordingly the relatedness was shown by dendrogram construction by DendroUPGMA. The grass samples were also used to check their antibacterial activity against potential pathogenic bacteria like *Pseudomonas aeruginosa, Salmonella typhi, Chrysobacterium gleum etc.* The plant species had showed a very satisfactory inhibition against the bacteria. *In vitro* anti-diabetic activity of *Cynodon dactylon* was checked by calculating its ability to inhibit the enzyme alpha-amylase and the anti-inflammatory activity was carried out by calculating the inhibition percentage of albumin degradation.

Outcome: All these experiments were done to study the pharmacological property as well as to gather knowledge on the genetic make-up of *Cynodondactylon.*Overall, the final outcome supports the ethanobotanical claim of this plant.

Keywords: anti-diabetic, anti-inflammatory, Bermuda grass, ISSR primers, dendrogram, polymorphism

1.INTRODUCTION

*Cynodondactylon*popularly known as the Bermuda grass [1] is a short-lived, prostrate and perennial grass. It grows on the soil very rapidly and forms a dense mat covering the soil. It is the main source of food for grazing cattle and many other herbivores. The culms of the plants are 5-45 cm long [2]. It is very much resistant to drought condition as the rhizome of the plant can survive by drought induced dormancy over a period of more than 7 months. After the drought is over and when normal conditions prevail it can again re-sprout with the help of stolons and rooted runners. Stolons and rhizomes play an important role in placement of leaves and roots in new microhabitats. In case of stolons and rhizomes their branching ability gets greatly reduced under lower light intensity and lower nutrient availability and branching ability is greatly accelerated under higher light intensity [3][4].It has the ability to withstand floods and also can revive after fire. It can control functions of the ecosystem by altering fire regimes, hydrological cycles, biophysical dynamics, nutrients cycles, and community composition [5]. Studies on normal rats and streptozotocin induced diabetic rats showed that aqueous extract of *Cynodon dactylon* exhibit antidiabetic effect. Among several doses 500mg/kg reduced 31% blood glucose level after 4 hour administration in normal rats and 23% in mild diabetic rats during Glucose Tolerance Test (GTT) [6]. It has high therapeutic and pharmacological effects as the phytochemical constituents of this species are flavanoids, alkaloids, glycosides, terpenoides, triterpenoids steroids, saponins, tannins, resins, phytosterols, reducing sugars, carbohydrates, proteins, volatile oils and fixed oils [7]. Genetic profiling or DNA fingerprinting of this grass species is done using ISSR primers. Inter simple sequence repeat or ISSR are widely used for genetic variation studies. It is used as no prior information on DNA sequence is not required for the primer and moreover their cost of synthesizing is low and can be used for any plant species.Genetic profiling using ISSR primers are done to study ploidy complex and often to know the geographic origin of some plant species. DNA profiling studies have been successfully used in analyzing relatedness of

Cynodon species includes DNA amplification fingerprinting (DAF), RAPD , AFLP, ISSR , SSR and chloroplast specific simple sequence repeat length polymorphism (CpSSRLP)[8].

The plant is highly useful in its medicinal properties. Aqueous extract of the whole plant as well as leaves and rhizome exhibit properties like Antipyretic, Analgesic, Antihelminthic, Anticataleptic, Antimicrobial, Antidiurectic and Anti-inflammatory. Thorough studies resulted that aqueous and ethanol extract of the whole plant showed antiepileptic and antidiabetic activity; hydro alcoholic extract of the aerial parts of the plant exhibited antioxidant activity and 50% aqueous ethanolic extract could reduce kidney stones. This plant could be used in cough, cramps, diarrhea, headache, warts, wounds, measles etc. as a traditional medicinal plant [9][10]. Our present study involves analyses of the genetic profiling of plant samples of 3 different species using ISSR primers and their dendrogram construction to understand the relatedness between the samples. Antidiabetic, antibacterial and anti-inflammatory activities were also investigated using leaf extracts.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Fresh green grass samples were collected surrounding GKVK, Bangaloreand stored. Three types of samples- Lawn grass (CD1), randomly available samples (CD2) and bigger leaflets (CD3) were taken for the genetic profiling studies.

2.2 Isolation of DNA

DNA was isolated from the fresh samples by CTAB method. 500 mg of the samples were ground with 1mL of pre-heated CTAB buffer [3% Cetyltrimethyl ammonium bromide, 5M NaCl, 0.5M EDTA (pH 8.0), 1M Tris-HCl (pH 8.0)]. The homogenate was obtained and incubated in dry bath at 60ºC for 30 minutes, and was cool down to room temperature after

completion of incubation. Chloroform and Isoamylalcohol in a ratio 24:1 was added in equal volume and gently mixed. It was then centrifuged in refrigerated centrifuge at 10,000 rpm for 10 minutes. After centrifugation the top layer was carefully transferred to another fresh tube and added equal volume of ice-cold isopropanol. The contents were centrifuged at 10,000 rpm for 10 minutes and the pellet was retained. 500μL of 70% Ethanol was added to the pellet and gently tapped to mix the pellet. It was then spun in refrigerate centrifuge at 10,000 rpm for 10 minutes. The pellet was collected and dried for about 45 minutes thereafter the pellet was mixed with TE buffer. Gel-electrophoresis was done to observe the desired isolated DNA.

2.3 Polymerase Chain Reaction and Electrophoresis

After gel-electrophoresis samples were subjected for PCR using random primers to understand the polymorphism between species. The PCR reaction mixtures and the primers used are as shown in Table 1 and Table 2. The initial denaturation was done at 94ºC for 1 minute followed by 40 cycles of denaturation at 92ºC for 2 minutes; primers were annealed at 35ºC for 1 minute and elongation at 72ºC for 2 minutes followed by extended elongation at 72ºC for 5 minutes. After amplification the products were electrophoresed for analysis on 1.5% agarose gel in 1X TBE buffer at 100V for 35 minutes.The gel was then visualized under UV transilluminator and photographed.

Table 1: Characteristics of the used primers in this study

SL.No.	PRIMERS	ANNEALING TEMPERATURE (°C)
1.	OPA 16	25
2.	OPB 11	25

3.	OPC 06	25
4.	OPC 16	25
5.	OPC 18	25
6.	OPD 13	27
7.	OPL 05	25
8.	OPW 04	25

Table 2: Composition of PCR reaction mixture

Components	Quantity (µL)
Primer	0.4
Template DNA	0.5
DNTP's	0.4
5X Buffer	1
Taq buffer	0.2
Nuclear free water	7.5

2.4 Dendrogram construction

A dendrogram is a hierarchical tree which shows clustering of genes or samples. Hierarchical clustering is a popularly used method for detecting clusters in genomic data. Clusters can be described as cutting branches off the Dendrogram[11]. It consists of data points which are connected by U-shaped lines. Dendrograms are of two types- column and row, both the types comprises of Clades and Leaves. Using the web server DendroUPGMA dendrograms were constructed based on UPGMA (Unweighted Pair Group Method using Arithmetic Mean) or WPGMA (Weighted Pair Group Method using Arithmetic Mean). UPGMA is a simple agglomerative hierarchical clustering method applied for the construction of dendrogram from distance matrix. The UPGMA method utilizes a sequential clustering algorithm, where

local topological relationships are inferred in descending order of similarity and a dendrogram is built in a gradual way. WPGMA is another algorithm that measues the distance between clusters is as a simple average [12]. Gel image was taken and bands were matched using PyElph software, binary matrix was generated. Using binary matrix Dendrogram was constructed through UPGMA method.

2.5 *In-vitro* antibacterial activity

Fresh leaves of *Cynodon dactylon* were collected from local shops in Bangalore, India to check its effect against urinary tract infection causing bacteria*Chryseobacteriumgleum*. From the leaf powder plant samples was extracted using the solvent ethanol. The plant extract was then used for testing antibacterial activity. It was done by agar well method where DMSO was used as negative control. Plant extracts were mixed with 2 concentrations of DMSO 100 mg/mL and 200 mg/mL and added to the wells made on the plate containing bacterial culture. Development of the zone of inhibitions was checked after 24 hours incubation at 37ºC.

2.6 *In-vitro* antidiabetic activity

From 10 grams of the powdered sample extract was prepared from ethanol for 24 hours. The extraction was done twice to make sure that we will not lose any phytocompounds. The extract was dried in a desiccator and used for testing *in-vitro* antidiabetic activity. In this method mixture of starch solution and plant extract was incubated at 25ºC for 10 minutes. After that 1 mL of 1% starch solution prepared in 2 mL phosphate buffer was added and incubated at 37ºC for 5 minutes to allow the reaction to happen. The reaction was terminated by the addition of 2 mL of 3,5 dinitro- salicylic acid reagent. Now the activity was measured by taking the absorbance at 540 nm with Glucobay 25 mg (1mg/mL). Inhibition of the enzyme α-amylase was noted in percentage. The formula for calculation of percentage of inhibition **I% = (Ac-As)/Ac X 100**

Ac= absorbance of control; As= absorbance of sample [13]

2.7 *In-vitro* anti-inflammatory activity

The above prepared methanol extract was used to estimate its *in-vitro* anti-inflammatory activity. The activity is determined by the inhibition of albumin denaturation. 5 ml of reaction mixture was prepared containing 0.2ml of bovine albumin, 2.8ml of phosphate buffered saline (PBS, pH 6.9) and 2ml of varying concentration of extracts (100-500 ppm). Equal volume of double distilled water was used as negative control. The reaction mixture was incubated at 37ºC for 15 minutes followed by heating at 70ºC for 5 minutes. Absorbance was observed at 660 nm using blank. Diclofenac sodium was used as the reference drug and treated for absorbance observation. The percentage of inhibition of protein denaturation was calculated. The formula for calculation of percentage of inhibition is $I\% = (Ac-As)/Ac \times 100$

Ac= absorbance of control; As= absorbance of sample [14]

3. RESULTS AND DISCUSSION

DNA whichwas isolated by CTAB method was further column purified to get rid of impurities and purity of the DNA was checked in spectrophotometer. The wavelength used was 260/280 nm and the concentration of DNA obtained was satisfactory to carry out other procedures. Table 3 shows the concentration and purity of isolated DNA when compared with standard 1kb ladder.The bands obtained for ISSR primers range from 60 bp to more than 100 bp and for CD1 7 bands, CD2 6 bands and CD3 4 bands were observed. Close observation of the gel revealed that each lane have bands which were similar with the other 2 bands though the morphology of the grass samples were different. This polymorphism of bands showed the variation among genotypes and this study would help in the investigation of other relatives of the specimen.Using the PyElph software similarity between the samples

was checked by band match (Figure 2).UPGMA by PyElph showed that CD1 and CD2 shared more similarity than that of CD3 (Figure 3). Dendrogram was constructed using the online tool DendroUPGMA and tree obtained was as shown in figure 4. Matrix was loaded in the online tool setting the parameters as similarity index used to compare between the variables - Jaccard index (Tanimoto) and clustering method – UPGMA (Unweighted Pair Group Method with Arithmetic mean) and then submitted. The results obtained depicted that the primers OPC06, OPC18, OPL05 and OPW04 have worked and the rest didn't work. The corresponding bands for p1, p2, p4 and p6 shared a common clade but didn't polymerize the product fragment in all plant specimens. While the bands for p7 and p3 sharing a common clade polymerized with CD1 and CD2 only and the bands for p5 and p8 shared a common clade as well as polymerized with CD1 only.

Table 3: Showing concentration and purity of isolated DNA

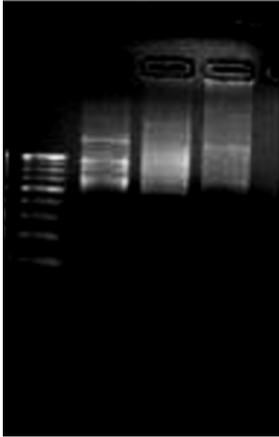| Sample-ID	Scientific name	260/280	Conc(ng/µl)
CD1	*Zoysia pacifica*	1.79	71.8
CD2	*Cynodon dactylon*	1.72	93.4
CD3	*Cynodon nlemfuensis*	1.77	184.1

Figure 1: Polymorphic bands on agarose gel for different primers
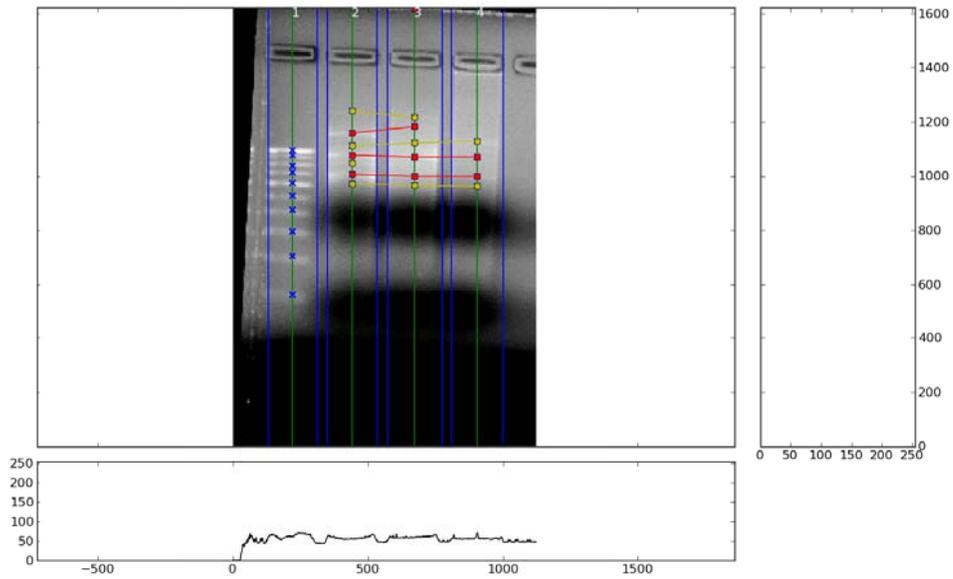

Figure 2: Band match using PyElph software [lane 1- ladder, 2- CD1, 3-CD2, 4-CD3specimens]
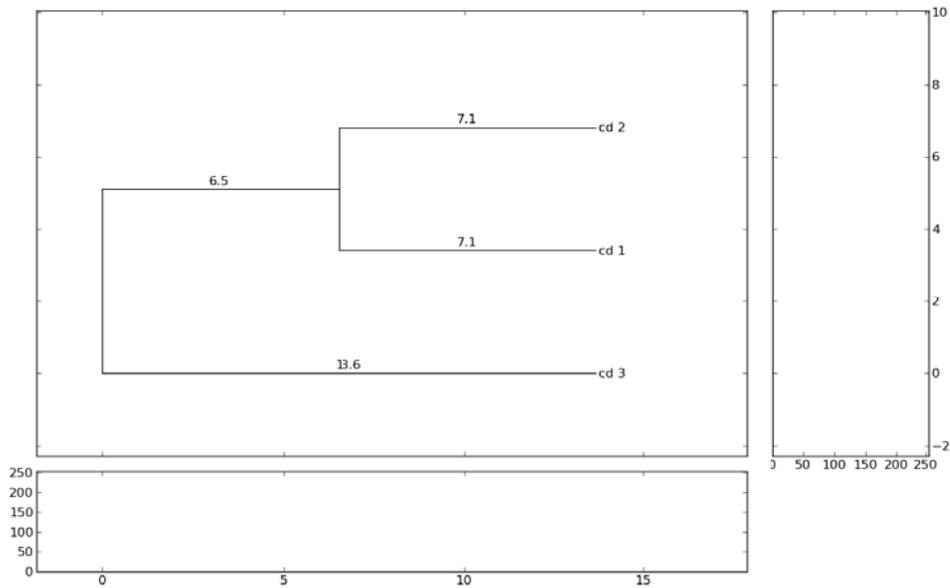
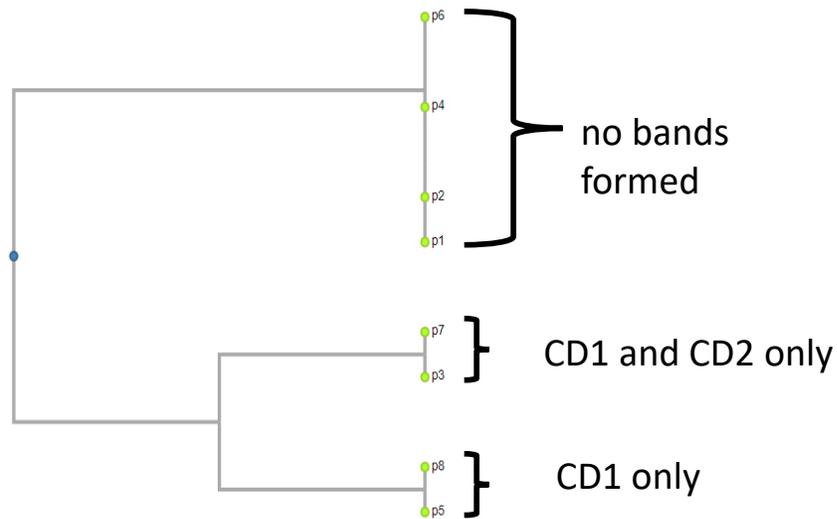**Figure 3: UPGMA results got by PyElph software**

Figure 4: UPGMA and cophenetic correlation coefficient results from the online tool

DendroUPGMA

Antibacterial Studies

The antibacterial property of the samples were tested on plates cultured with

Chrysobacterium gleum, Klebsella pneumoniae, Pseudomonas aeruginosa, Pseudomonas

syringae, Salmonella typhi and *Xanthomonas campestris*. All these bacteria are gram negative, pathogenic and cause disease in either in plants or animals the most lethal among these is *Pseudomonas aeruginosa* which is a multi-drug resistant bacteria as well as an antibiotic resistant [15] one while *Pseudomonas syringae* is a very interesting bacteria as it is an atmospheric "biological ice nucleator" which can freeze the water of leaves and cause an injury. It is a potential plant pathogen and acts as model to study host-microbe interactions, bacterial virulence mechanisms, host adaptation of pathogens, as well as microbial evolution, ecology and epidemiology [16] *Chrysobacterium gleum* is a gram-negative, non-fermenting bacteria found widely in soil and water. It is an emerging pathogen and can be acquired through nosocomial infection. It is a metallo β-lactamase producing bacterium [17][18].This bacteria is yellow pigmented on blood agar, catalase positive, oxidase positive, non-motile, non-glucose fermenting Gram negative bacilli on MacConkey agar. *Chryseobacterium* spp are resistant to several antibiotics such as aminoglycosides, chloramphenicol, tetracyclines, clindamycin, teicoplanin, and erythromycin [19].

Antibacterial studies revealed that *Cynodon dactylon* extract has significant effect on *Chrysobacterium gleum* forming a zone of inhibition with 3 mm diameter in 200 mg/ml concentration. It has also affected the other bacteria which are as tabulated in Table 4.

Table 4: Respective microorganism and inhibition zone formed by extract dissolved in DMSO

Micro organism	Gram staining	Inhibition zone (mm)	
		1 (200mg/mL)	2 (100mg/mL)
Chrysobacterium gleum	negative	3	1
Klebsella pneumoniae	negative	4	1
Pseudomonas aeruginosa	negative	1	3

Pseudomonas syringae	negative	1	3
Salmonella typhi	negative	2	1
Xanthomonas compertris	negative	0	0

Antidiabetic Studies

A graph was obtained by calculating the inhibition percentage of α-amylase. The control used for this study Glucobay has an active amylase inhibition ingredient called Acarbose which is a synthestic chemical. 1 mg/ml concentrated Acarbose showed 79% inhibition rate and 400 ppm of *Cynodon dactylon* extract showed 72%. This result opened a new modus operandi for antidiabetic drug synthesis as *Cynodon dactylon* is a plant which can act as a superb alternative to synthetic drugs as shown in figure 5.
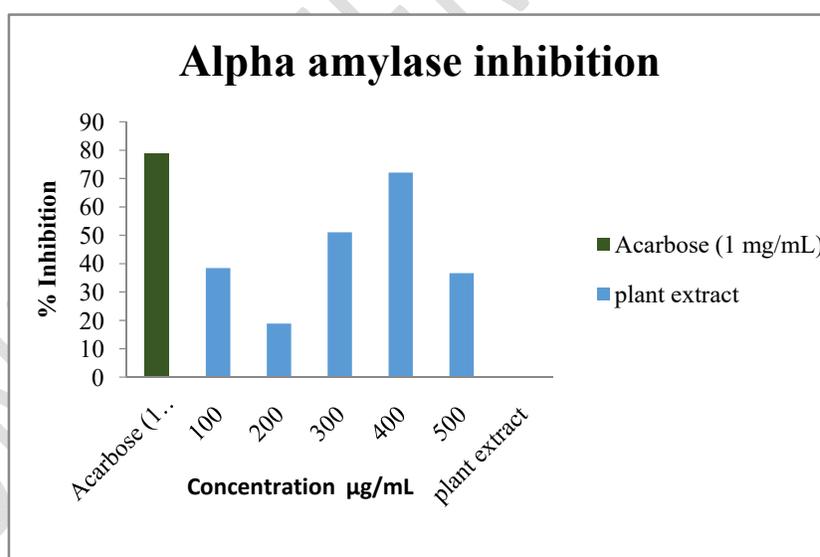
Anti-inflammatory Studies

Anti-inflammatory activity is based on the capability of inhibiting albumin degradation. The graph obtained by calculating the percentage showed that with increasing concentration of the

plant extract capacity of inhibiting albumin degradation also increased. The control Sodium diclofenac, a standard drug showed 79 % inhibition rate at 1mg/ml concentration and plant extract with 500 ppm of concentration showed 62% of inhibition rate as shown in figure 6.
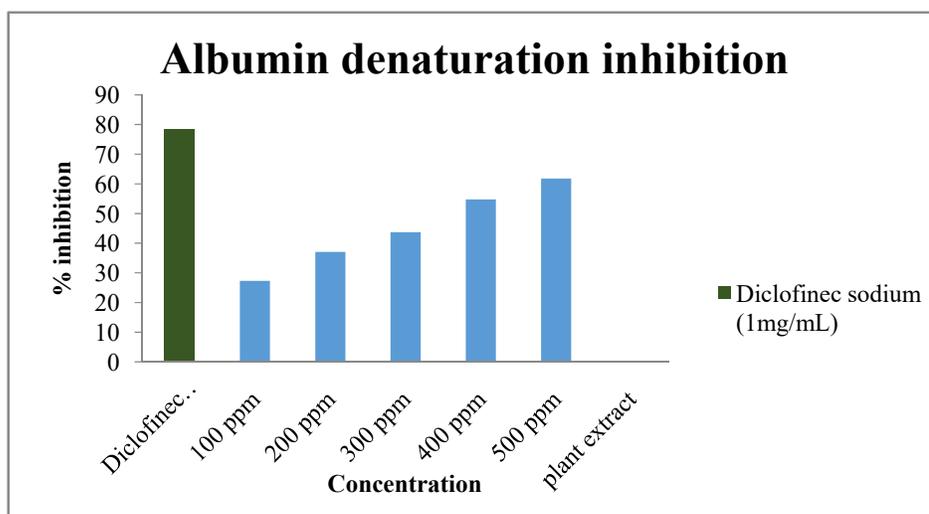

Figure 6: Albumin denaturation inhibition showed by *Cynodon* extract

*Cynodondactylon*is a medicinal plant and is used to treat inflammations in traditional medicine. In a study doses ranging from 200, 400 and 600 mg/kg of the aqueous extract of the plant was administered on carrageenan, serotonin, histamine and dextran induced rat paw edema. The study concluded that all the doses are safe when taken orally and considerably reduced ($p < 0.001$) the edema formation induced by carrageenan, serotonin, histamine and dextran after 3 and 5 hours. [20] The plant exhibits antidiabetic activity and can be used as an alternative. Studies had shown that aqueous, chloroform, ethanol and methanol extracts when treated on alloxan induced diabetic rats considerably reduce blood glucose level. Among all these solvents methanol extract had reduced the blood glucose level of the diabetic rats to 108 ± 10.0 mg/100ml which is similar to that of the normal rats. [21] In another study it is observed that *Cynodon dactylon* has antibacterial activity. It was observed that aqueous and chloroform

extract of this plant inhibited *Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli* and *Klebsiellapneumoniae*[22].In this study also it was observed that *Cynodondactylon*possess antibacterial activity showing immense sensitivity to *Chrysobacteriumgleum*. It also had anti-diabetic and anti-inflammatory property when checked with respect to acarbose and diclofenac sodium respectively.

A species without enough genetic diversity is thought to be unable to cope with changing environments or evolving competitors and parasites [23]. In addition, the ability of a population to respond adaptively to environmental changes depends on its level of genetic variability or diversity [24]. Thus, genetic diversity in indigenous breeds is a major concern considering the necessity of preserving what may be a precious and irreplaceable richness, regarding new productive demands. Conservation should be based on a deep knowledge of the genetic resources of the specific breed. Therefore, it is important to try to genetically characterize indigenous breeds [25] and the applications of molecular genetics have many important advantages [26]. The ISSR is a molecular marker method which does not need the genome sequence information and leads to multi-loci and highly polymorphic patterns [27]. Each ISSR band corresponds to a DNA sequence delimited by two inverted microsatellites. The ISSR loci are dominant markers with the assumption of only two alleles per locus. It has been shown that the ISSR markers are universal, quick, easy to apply, highly reproducible and polymorphous [28]. The ISSR method has been used in genetic diversity studies in several species such as cattle [29], cattle, goat and sheep [27], sheep [28], fish [30], silkworm *Bombyxmori*[31] et al., 1999, mouse [32]and has proved to be universal.

4.CONCLUSION

Cynodondactylon or Bermuda grass rapidly grows in warm climate and forms a dense mat by underground rhizomes and surface stolons. Since it is a lawn grass it needs regular mowing

and if left unmowed can reach up to 2 feet high. It is a C4 plant and has various useful properties. This study is very useful in understanding the pharmacological property of the plant. Since the study showed that this plant has active anti-diabetic activity it can be used as a potential alternative against synthetic compounds moreover it has antibacterial activity and can be used to synthesize antibiotic to treat potential pathogens. This study is very promising and will surely unveil new methods of treatment in themedical world.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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