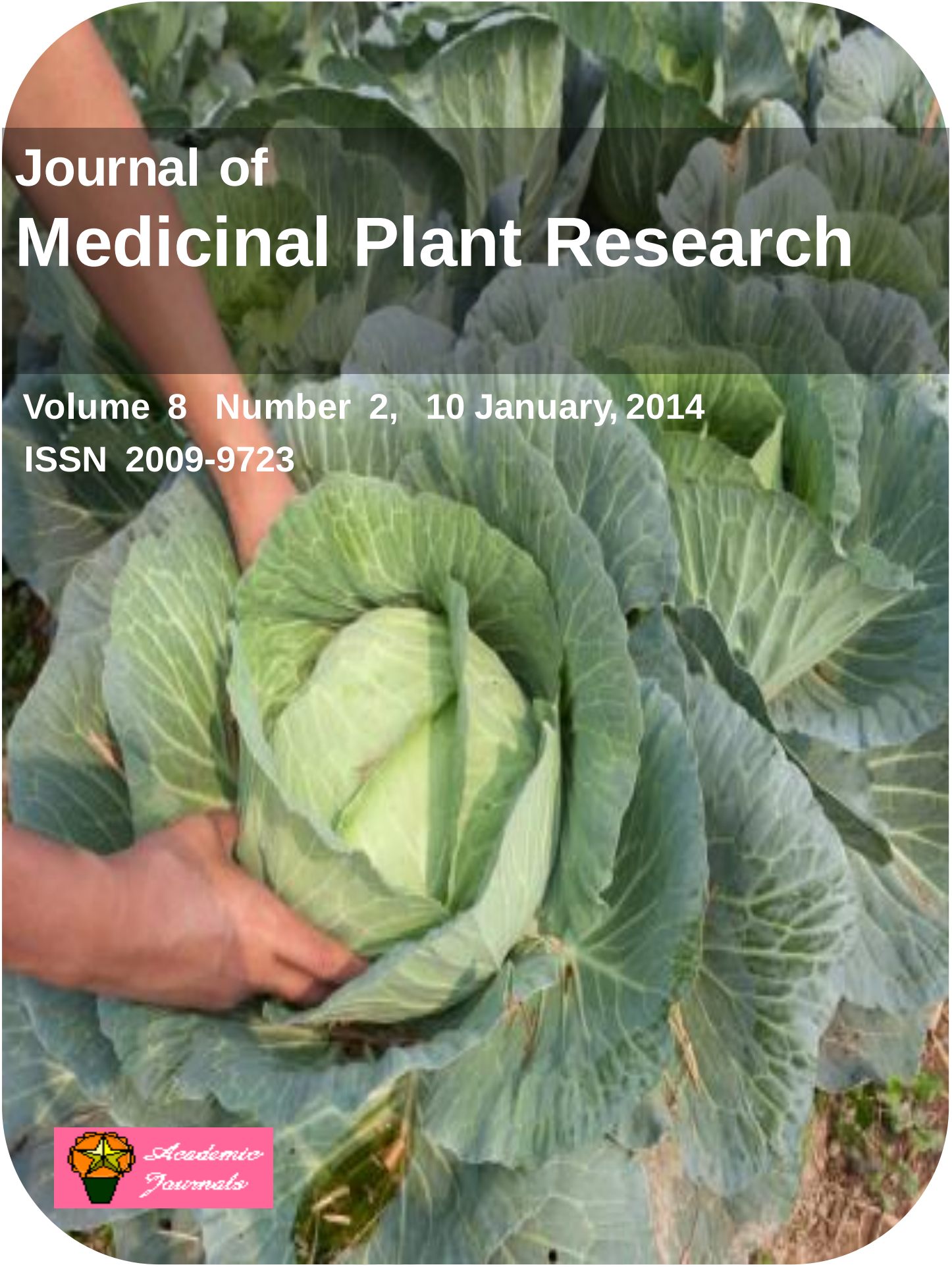

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Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001)
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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Research Articles

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Full Length Research Paper

Comparative assessment of genetic diversity among the Asclepiadaceous species using randomly amplified polymorphic DNA (RAPD) markers and numerical taxonomy system (NTSYS) cluster analysis

Adeeba Tariq*, Tariq Mehmood, Mushtaq Ahmad, Uzma Sahar, Shazia Mushtaq and Muhammad Zafar

Department of Plant Scences, Quaid-I-Azam University, Islamabad, Pakistan.

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Asclepiadaceae is an economically important family species which are the source of fiber, rubber and dyes. In this study, genetic variability has been determined in three species, that is, *Tylophora hirsuta, Wattakaka volubilis* and *Cryptolepis buchananii.* The aim of present study was to understand the extent and pattern of genetic diversity among the individuals of same and different species of Asclepiadaceae. To asses the level of polymorphism within the species and members of different species, randomly amplified polymorphic DNA (RAPD) markers were used. Sixty RAPD primers of OPA, OPC, OPF and OPG series were used; only eight primers of OPC series gave amplification. Maximum polymorphism at interspecific and intraspecific levels was shown by OPC 09 and minimum polymorphism was observed in OPC 05. The data was analyzed using software numerical taxonomy system (NTSYS) cluster analysis PC version 2.20. In total 190 monomorphic and 78 polymorphic bands were produced from all primers. Therefore, out of 322 amplified products, 59% were monomorphic and 24.22% were polymorphic. Low genetic diversification was found both at intraspecific and interspecific level. Mixed pattern of grouping in the analyses indicated the close affinities of species with each other.

Key words: Comparative assessment, genetic diversity, Asclepiadaceous species, randomly amplified polymorphic DNA (RAPD) markers on Asclepiadaceae.

INTRODUCTION

Asclepiadaceae have 175 to 180 genera and 2000 species which are distributed mainly in the tropical and subtropical regions of the world. In Pakistan, it is represented by 23 genera and 41 species. *Tylophora* have 50 species, represented in Pakistan by 2 species, namely, *Tylophora hirsuta* and *Tylophora tenerrima* (Nasir and Ali, 1983). There are two species of *Wattakaka* in Pakistan, *Wattakaka volubilis* and *Asclepias volubilis*. Genus *Cryptolepis* have 12 species. Only one species is known from Pakistan, which is *Cryptolepis buchananii* (Nasir and Ali, 1983). Members of Asclepiadaceae are distributed in paleotropical regions. It is distributed throughout India, Nepal, Cylon, Malaysia, South China, tropical Asia and Africa. In Pakistan, they are distributed in North Punjab, Azad Kashmir, Hazara, Rawalpindi, Attock and salt range (Nasir and Ali, 1983). Members of this family are mostly herbs, lianas, shrubs or trees (rarely) which are laticiferous. Normal plants

*Corresponding author. E-mail: adeeba.tariq@gmail.com.

have very peculiar vegetative form. Some plants are cactoid; with leaves modified as pitchers in *Dischidia rafflesiana.* They are well developed or much reduced, opposite (decussate, usually) or whorled (rarely and rarely spiral) and fleshy or membranous or modified into spines. Lamina is entire (often reduced) containing one-vein or pinnate or palmate. They are stipulate or exstipulate (Watson and Dallwitz, 1992). The basic chromosome number x = 11 is the most predominant number in Asclepiadoideae, Periplocoideae and Secamonoideae. Only 3.5% of the studied species and taxa deviate from this basic chromosome number. Among the deviant ones, x = 10 is the most frequent followed by x = 9 and the sporadic increasing deviates x = 12, 13, and 14 (Albers and Meve, 2001).

Molecular markers have been looked upon as tools for a large number of applications ranging from localization of a gene to improvement of plant varieties by marker-assisted selection. They have also become extremely popular markers for phylogenetic analysis adding new dimensions to the evolutionary theories (Joshi et al., 1999). Restriction fragment length polymorphism (RFLP), DNA sequencing, and a number of polymerase chain reaction (PCR) based markers are being used extensively for reconstructing phylogenies of various species. The techniques are speculated to provide path-breaking information regarding the fine time scale on which closely related species have diverged. Furthermore, these studies hold a great promise for revealing more about the pattern of genetic variation within species (Avise, 1994). Coding sequences played an important role, however, non coding sequences are also an important resource for phylogenetic reconstruction. There are six different kinds of nuclear DNA marker systems: (1) internal transcribed spacer (ITS), (2) external transcribed spacer (ETS), (3) simple sequence repeats (SSRs), (4) transposable elements (TE), (5) nuclear introns, and (6) promoter regions. Among them, the two most widely used non coding DNA marker systems are the nuclear encoded ITS (Internal Transcribed Spacer of nuclear encoded RNA) and the plastidic *trnLF* region (*trnL intron, trnLF* intergenic spacer) (Calonje et al., 2008).

As very little work has been done on the molecular aspect of *T. hirsuta, C. buchananii* and *W. volubilis,* the need was felt to explore and study the genetic variations of these plants and their taxonomic status at molecular level. The present thesis aims to study the pattern of genetic diversity in *T. hirsuta, C. buchananii* and *W. volubilis,* using RAPD markers in order to establish polygenetic relationship among these genera.

MATERIALS AND METHODS

Plants of three different genera of Asclepiadaceae *(Tylophora, Cryptolepis, Wattakaka*) were collected from Quaid-i-Azam University campus Islamabad. Young leaves were collected and stored at 4°C in sealed plastic bags. Total genomic DNA was extracted from fresh leaves by CTAB method (Richards et al., 1997) with few modifications. Two to three leaves of the plant material were ground with the help of pestle and mortar, by adding 1 ml of preheated (65°C) 2X Cetyl trimethyl ammonium bromide (CTAB) buffer. All the purified DNA samples were stored at -20°C for further use. The presence of DNA and its quality was checked by running it on 1% agarose gel prepared in 0.5X Tris acetate ethylene diamine tetra acetic acid (TAE) buffer.

The concentration of DNA was measured with the help of spectrophotometer at 260 nm wavelength and ratios of OD_{260}/OD_{280} were recorded. The ratio 1.8 to 2.0 was taken as an indication of good quality DNA (Pich and Schubert, 1993).

The amount of DNA was quantified using the formula:

$$\text{DNA concentration } (\mu g/ml) = \frac{O.D_{260} \times 100 \text{ (dilution factor)} \times 50}{1000}$$

Sixty primers of different RAPD series (Gene link) were used to analyze the genetic variation among the sample plants. These primers were from OPA, OPC, OPF and OPG series. Only the random primers of OPC series gave amplification profiles. Ten random primers of OPC series were tested in RAPD analysis, namely, OPC 01, OPC 02, OPC 03, OPC 04, OPC 05, OPC 06, OPC 07, OPC 08, OPC 09, and OPC 10. Scoreable amplification profiles were given by all the aforementioned primers except OPC 03 and OPC 10. The sequence of ten primers (OPC series) is given as shown in Table 1.

Table 1. The sequence of ten primers (OPC series).

Primer	Base sequence (5´-3´)
OPC1	TTCGAGCCAG
OPC2	GTGAGGCGTC
OPC3	GGGGGTCTTT
OPC4	CCGCATCTAC
OPC5	GATGACCGCC
OPC6	GAACGGACTC
OPC7	GTCCCGACGA
OPC8	TGGACCGGTG
OPC9	CTCACCCTCC
OPC10	TGTCTGGGTG

Different PCR conditions were used for the optimization of amplification, however, the best suitable conditions were as follows: initial denaturation at 94°C for 1 min followed by 44 cycles of denaturation at 94°C for 30 s, annealing at 40°C for 1 min and extension at 72°C for 2 min. Final cycle was same except extension for 7 min at 72°C. After PCR, contents were held at 4°C till use. The genomic DNA was amplified by selected primers in 25 µl reaction mixture containing 25 to 50 ng/µl DNA, 25 pmol primer, 12.5 µl 2x PCR master mix (Fermentas) and 10.5 µl of PCR water (Fermentas) using thermal cycler (Labnet, multigene gradient).

Amplified products were run on 1.5% agarose gel run in 0.5X TAE buffer. Gel documentation was carried out by Dolphin Doc Plus gel documentation system (Wealtech). The size of each band was estimated by using 100bp DNA ladder plus (Fermentas). Bands were recorded as present (1) or absent (0). Cluster analysis was carried out based on similarities within class and dissimilarities between different classes, that is, samples showing good correlation have been placed in same group. All monomorphic and polymorphic bands were scored and included in the analysis.

Table 2. Total number of bands produced from six primers of OPC series.

S/N	Lane No.	OPC 04	OPC 05	OPC 06	OPC 07	OPC 08	OPC 09	Total bands
1	1	5	5	3	3	2	3	21
2	2	5	7	5	3	3	3	26
3	3	5	6	5	3	2	2	23
4	4	5	5	5	3	3	3	24
5	5	5	4	4	3	3	3	22
6	6	5	2	2	3	1	4	17
7	7	5	4	4	3	4	3	23
8	8	5	1	4	3	4	7	24
9	9	5	1	3	3	3	6	21
10	10	5	2	2	3	3	3	18
11	11	5	5	3	3	4	2	22
12	12	5	5	2	3	3	3	21
13	13	5	6	2	3	4	1	21
14	14	5	5	4	3	1	2	20
15	15	5	5	1	3	2	3	19
Total		75	63	49	45	42	48	322

Presence or absence of unique and rare bands was used to generate genetic similarity coefficients and then similarity coefficients were used to construct dendrograms by computer programme (NTSYS) pc version 2.20 (Rohlf, 2002).

RESULTS AND DISCUSSION

Genomic DNA was isolated from leaves of *T. hirsuta, C. buchananii* and *W. volubilis*. Isolated genomic DNA was run on 1% agarose gel after treatment with RNase A for determining the quality and quantity of DNA. In PCR, high quality of DNA is required for amplification purpose; therefore, good quality DNA was confirmed by agarose gel electrophoresis. By using this genomic DNA as a template, amplification was carried out by using different RAPD primers of OPC series. Amplified products were confirmed by running it on 1.5% agarose in 0.5X TAE at constant voltage.

The size range of all the amplified fragments produced by the aforementioned primers was 200 to 1200 bp. In total, 322 bands were produced by all the primers used in this study. Among these 322 bands, 190 monomorphic and 78 polymorphic bands were observed (Figure 1). Therefore, out of 322 amplified products, 59% were monomorphic and 24.22% were polymorphic with an average of 11% polymorphism per primer. Maximum number of bands was produced by OPC 04 and minimum number of bands was produced by OPC 08. In one lane, maximum number of seven and minimum number of one band was found. Maximum polymorphism was observed in OPC 09 and minimum polymorphism was observed in OPC 05. However, no polymorphic band was observed in OPC 04 and OPC 07, therefore have shown 100% similarity (Table 2).

Cluster analysis of bands produced by all primers

Similarity indices were developed on the basis of amplified products of six RAPD primers with samples of three different species (*T. hirsuta*, *C. buchananii* and *W. volubilis*). These similarity coefficients are shown in Table 2. The range of genetic similarity values were from 0.42 to 0.85 with the mean of 0.63. The lowest similarity value was present between the sample number 2 and 8, while the highest similarity value was found between sample number 2 and 5. UPGMA cluster analysis has also revealed the same results. In cluster analysis of all primers sample number, 2 and 5 were 100% similar while sample number 2 and 8 had shown very low similarity level. Both of them (2 and 8) were so distant from each other that they were present in different clusters.

UPGMA cluster analysis of all primers revealed two major clusters in this cladogram (Figure 2). Data from six primers of OPC series were analyzed in this cluster analysis and samples had showed 63% similarity level and 37% divergence among them. Cluster 1 was characterized by ten samples (1, 2, 3, 4, 5, 11, 12, 13, 14 and 15). All samples of *T. hirsuta* (1, 2, 3, 4 and 5) were clustered together in cluster 1. All samples of *W. volubilis* (11, 12, 13, 14 and 15) were also clustered together in cluster 1. 70% similarity level was present in all the samples of cluster 1. Cluster 1 was further divided into subcluster 1 and subcluster 2. Subcluster 1 contained all samples of *W. volubilis* and they had shown 74% similarity. Two groups were present in subcluster 1, that is, G 1 and G 2. Sample number 14, 15 and 11, 13 were clustered in groups 1 and 2, respectively. Both of them had shown 84% similarity. These were the least diverse samples of *W. volubilis*. Sample 12 was closely related to

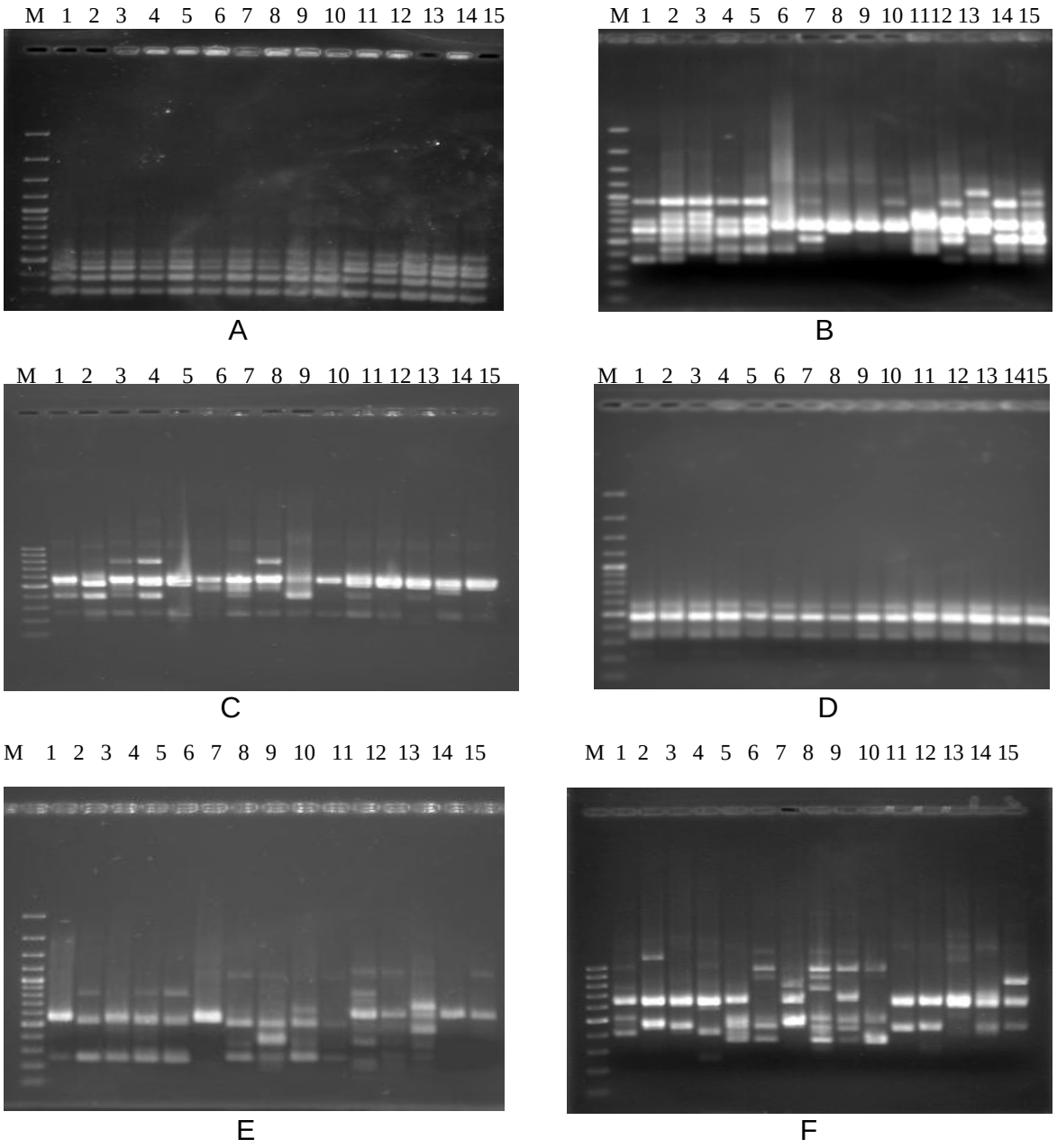

Figure 1. RAPD amplification profiles of fifteen samples of different species with different primers. A: OPC 4, B: OPC 5, C: OPC 6, D: OPC 7, E: OPC 8, and F: OPC 9. M: marker (DNA ladder), *Tylophora hirsuta* (sample 1-5), *Cryptolepis buchananii* (sample 6-10) and *Wattakaka volubilis* (sample 11-15).

group 1, but it was unresolved. It means that its origin was not clear. Only one sample of *T. hirsuta* (1) was present in subcluster 1, but it was also unresolved.

In subcluster 2, four samples of *T. hirsuta* were clustered. They had shown 80% similarity with each other. One group was present in subcluster 2 (G 3). Sample numbers 2 and 5 were clustered together in group 3, because they had shown 100% with each other. Samples 3 and 4 were unresolved but closely related to group 3. Sample 1 was the most diverse one among all the samples of *T. hirsuta*.

Cluster 2 was characterized by five samples of *C. buchananii* (6, 7, 8, 9 and 10). They had shown 72% similarity. A group was formed in cluster 2, that is, G 4, which was characterized by two samples, that is, 9 and 10. They were the least diverse samples of *C. buchananii*, because they were at 84% similarity level. The other three samples 6, 7 and 8 were unresolved. Among the samples of *C. buchananii*, sample 6 was the most diverse sample. UPGMA cluster analysis had shown a clear picture about the position of three genera. Presence of *T. hirsuta* and *W. volubilis* in a same cluster

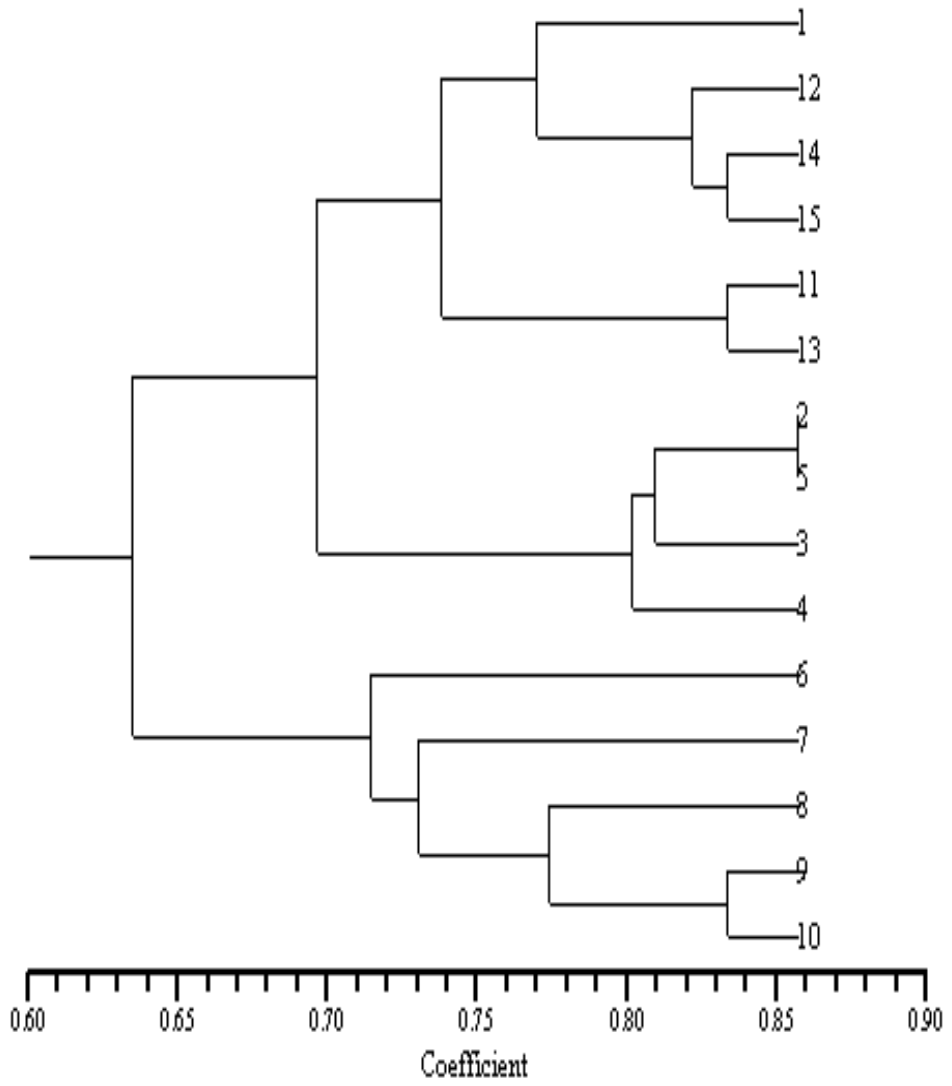

Figure 2. Dendrogram produced by amplified products of all primers. The number corresponds to the species as follows: *Tylophora hirsute* (1, 2, 3, 4, 5), *Cryptolepis buchananii* (6, 7, 8, 9, 10), *Wattakaka volubilis* (11, 12, 13, 14, 15). C: cluster, Sc: subcluster, G.

had revealed their close relationship with each other. Position of sample 1 of *T. hirsuta* in dendrogram with the samples of *W. volubilis* showed very close relationship of this sample with *W. volubilis.*

Cluster analysis of all primers has shown that genetic diversity is present within the species and between different species. Species are the units of ecological diversity and alleles are the units of genetic diversity. Samples of different species simply harbor more distinct alleles than samples of the same species. Weighting of ecological measures of species diversity on the basis of their genetic distance has been considered by several authors (May, 1990; Humphries et al., 1995). Individuals within a given species share some percentage of alleles; otherwise, they would not be considered members of the same species. This shared (or common) portion of the gene pool includes two basic classes of genes. Genes that are monomorphic within species are common to all individuals. The remainder of loci (on average, 15% in animals and 50% in plants) is polymorphic, varying among individuals in a population, and among populations within a species. The genetic profile of whole populations typically varies from place to place across a species range. These differences may arise as the result of chance occurrences, such as the genetic composition of dispersing individuals that create a new population (founder effect), or changes in allele frequencies that result from chance mating in very small populations (genetic drift) (Primack and Kang, 1989; Templeton, 1991; Meffe and Carroll, 1994; Eckert et al., 1996; Husband and Schemske, 1996).

Differences among species can also arise systematically,

especially if the environment in various places exposes individuals to different optima for survival and reproduction. For these and other reasons, species often diverge from one another in their genetic composition (Hartl and Clark, 1997). Each species distributes its genetic diversity (one measure of which is the total of all alleles at all loci) in a pattern reflecting both its biology and its history (Wright, 1965; Nei, 1975). Samples of two different species, that is, *T. hirsuta* and *W. volubilis* have shared some percentage of common alleles, that is why they are present in same cluster (Figure 2). These results also indicate that these two species have interspecific variations. Intraspecific variations have also been observed (Figure 2), like the individual of *T. hirsuta* (1) which is more diverse than other individuals of same species. However, this sample is more closely related to the samples of other species, that is, *W. volubilis.* Evidences from other studies had shown that within species variation might be present like in *Hoya parasitica* (Asclepiadaceae) which is one of the most common members of the section Euhoya (Hooker, 1883). *H. parasitica* is the most common, relatively widespread and extremely variable species (Rintz, 1978). Taxonomic study of the species (Kiew, 1995), included variations among the individuals of *H. parasitica.* There were variation in texture, shape, size and venation of leaves, the size of the flower and shape of corolla lobes. From these studies it was concluded that *H. parasitica* complex is composed of several undescribed taxa (Kidyue et al., 2005).

Variations might be present within the individuals of same species collected from same geographic area. Studies have indicated that all samples of Asclepiadaceae which were collected from Western North America had shown genetic variations among them. Some evolutionary lines vary less than others and more importantly, all the members of a particular line from one geographic area will often vary approximately in the same way (Stebbins, 1974). While the Asclepiadaceae were sampled more southerly in distribution, with only a few exceptions, all were taken from areas north of the Tropic of Cancer and must have been subjected to many of the same types of climatic shifts during the Tertiary and Quaternary Periods. In addition to the effect of similarity in geographic range, a considerable amount of variations within the species or between different species is observed (Gilmartin, 1980).

All three species which are studied in this research project share same habitat and occur sympatrically. *T. hirsuta* and *W. volubilis* both are from tribe Marsdeieae. It was evident that members of Asclepiadaceae are insect pollinated. This could have affected the evolutionary processes to produce the resulting like-patterns of the overall variability within the constituent species and taxa. Studies had revealed that tribes Marsdenieae and tribe Asclepiadeae are the most prominently *Diptera* serviced groups (Ollerton and Liede, 1997). Variations might be present at interspecific levels due to the same insects, during the process of pollination. In cluster 2 (Figure 2), sample of *C. buchananii* have shown a narrow range of diversity among them. Same is the case with the samples of *W. volubilis* in cluster 1. This low range of diversity is due to same soil and climatic conditions. *C. buchananii* is the member of sub family Periplocoideae and *T. hirsuta* and *W. volubilis* are the members of Asclepiadoideae. All the members of three species have 63% similarity among them. These results indicate that all the members are related to each other and have shown low genetic diversification. During the course of evolution, they have adapted to the most suitable environmental conditions, this might be the cause of their genetic divergence.

Cladistic analyses using morphological (Judd et al., 1994; Struwee et al., 1994) and molecular (Civeyrel, 1996; Civeyrel et al., 1998; Sennblad, 1997; Sennblad and Bremer, 1996, 2002; Sennblad et al., 1998) data have supported the contention that Asclepiadoideae and Periplocoideae form monophyletic tribes. In a study involving 15 taxa from the *Apocynacs* and 22 taxa from the *Asclepiads,* the three main subdivisions within the *Asclepiads*: Periplocoideae, Secamonoideae and Asclepiadoideae were supported (Civeyrel et al., 1998). In addition, the *Asclepiads* formed a monophyletie group (Potgieter and Albert, 1998b; Potgieter, 1999), and there is even some indication that the *Asclepiads* may be biphyletic. Civeyrel et al. (1998) reported the monophyly of the family Asclepiadaceae and of the three subfamilies, Periplocoideae, Secamonoideae, and Asclepiadoideae. Cluster analysis of *T. hirsuta, W. volubilis* and *C. buchananii* have also shown the mixed pattern of grouping among the individuals. These results indicate that these species have a very close affinity with each other. This is because they belong to a same family. These three genera belong to the two different subfamiles within Asclepiadaceae. *T. hirsuta* and *W. volubilis* are present in subfamily Asclepiadoideae and *C. buchananii* is present in subfamily Periplocoideae. Our results have also proved that *T. hirsuta* and *W. volubilis* which are present in same cluster and showed a close relationship, belonged to the same subfamily, even of the same tribe, that is, Marsdenieae. Species of this cluster have shown close affinities with each other. *C. buchananii* belonged to different subfamily, that is, Periplocoideae, and in our analysis, it is also present in different cluster.

Conclusion

Genetic variations among three species of different genera of Asclepiadaceae (*T. hirsuta, C. buchananii* and *W. volubilis*) were analyzed. For this purpose, different series of RAPD marker had been used to study the polymorphism among the species of three genera and also among the samples of same species. Results have

shown that low genetic diversification is present at interspecifc and intraspecific levels. Species have shown mixed pattern of grouping with each other while using different RAPD primer. Samples of *T. hirsuta* and *W. volubilis* are grouped together in one cluster, while samples of *C. buchananii* are grouped together in the second cluster. These interspecific relationships indicated that three genera belong to two subfamilies of Asclepiadaceae, that is, Asclepiadoideae and Periplocoideae. High level of similarity at intraspecific level has also shown that all the samples of each species are monophyletic. In future, if this work has been done on broader spectrum, then a complete gene pool of Asclepiadaceae would be developed. New species and varieties could be discovered. It would also help us to do an authentic and synthetic characterization of Asclepiadacea

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Medico-ethnobotanical investigations in Parbat district of Western Nepal

B. Malla[1]*, D. P. Gauchan[2] and R. B. Chhetri[1]

[1]Department of Environmental Science and Engineering, Kathmandu University, P.O. Box No: 6250, Dhulikhel, Kavre, Nepal.
[2]Department of Biotechnology, Kathmandu University, P.O. Box No: 6250, Dhulikhel, Kavre, Nepal.

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The aim of present research was to record the indigenous knowledge on medicinal plants of Parbat district, Nepal. Field trips were arranged to collect the plants and ethnobotanical information from the study area during 2011 to 2012. The paper dealt with ethnobotanical plants along with their local names, parts and ethnomedicinal uses prescribed by ethnic tribes Gurung, Magar and Majhi of the district. A total of 61 plant species belonging to 59 genera and 43 families had been used by the local tribes for curing various human diseases. The plant specimens were also collected dried, pressed, mounted on herbarium sheets and deposited to the Department of Environmental Science and Engineering, Kathmandu University. The investigation provided immense scope for the active principle analysis and clinical studies of these ethnomedicinal plants for future drug development.

Key words: Ethnobotany, medicinal plants, traditional healers, Gurung, Magar, Majhi.

INTRODUCTION

In Nepal, there are about 8.4 million indigenous nationalities of different groups inhabiting various terrains. These indigenous people possess their own culture, religious rites and rich traditional medicine practices. Those ethnic people residing in different geographical belts of Nepal depends on wild plants to meet their basic requirements and all the ethnic communities have their own pool of secret ethnomedicinal and ethnopharmacological knowledge about the plants available in their surroundings (Bhattarai et al., 2006a; Ghimire and Bastakoti, 2009; Joshi et al., 2011; Kunwar et al., 2009; Manandhar, 2002; Shrestha and Dhillion, 2003; Acharya and Acharya, 2009; Bhattarai et al., 2009; Rokaya et al., 2010; Uprety et al., 2010; Panthi and Chaudhary, 2003) which has been serving rural people with its superiority.

Due to changing life style, extreme secrecy of traditional healers and negligence of youngsters, the practice and dependence of ethnic societies in folk medicines is in rapid decline globally, therefore, ethnobotanical exploitation and documentation of indigenous knowledge about the usefulness of such a vast pool of genetic resources is deliberately needed (Bussmann and Sharon, 2006; Behera and Mishra, 2005; Rajkumar and Shivanna, 2010; Saikia et al., 2006; Rana et al., 2010). Traditional medicine in Nepal comprises those practices based on beliefs that were in existence often for hundreds to thousands of years before the development and spread of modern medicine, and which are still in use today (Hamilton and Radford, 2007). Long ago, there is a common practice of using plants in traditional medicine in Nepal, because it is not only easily available, but also affordable to the rural people (Manandhar, 1998). According to WHO (2001), 80% of the world population or roughly two thirds of the world's population, rely almost exclusively on traditional medicines using natural substances mostly derived from plants in the treatment of diseases. Approximately 90% of

*Corresponding author. b.malla@nins.edu.np.

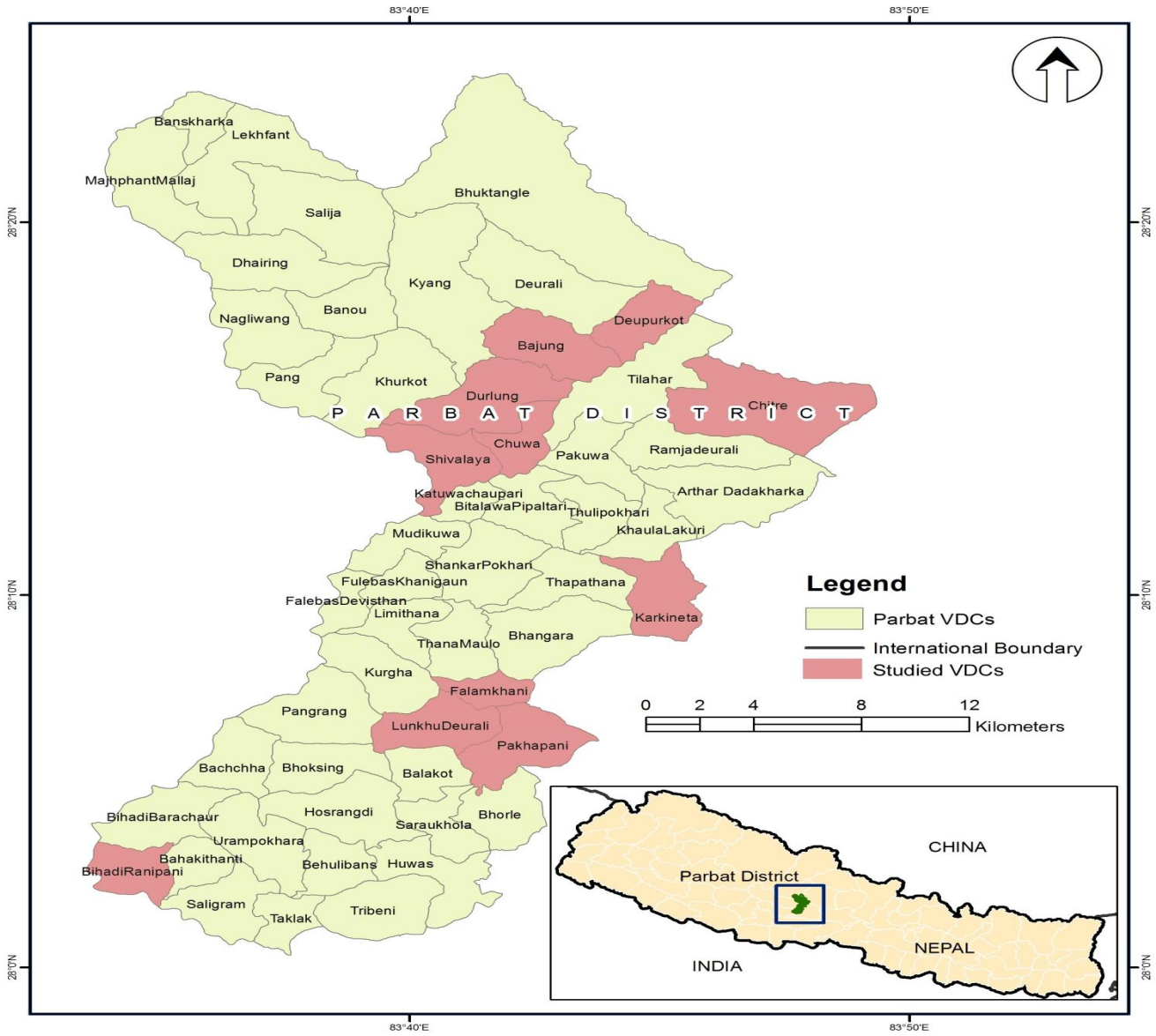

Figure 1. Map showing study area.

the Nepalese people reside in rural areas where access to government health care facilities is lacking (Bhattarai, 1998).

Investigation and documenting traditional knowledge through the ethnobiological approach is important for highly valuable medicinal plant conservation and sustainable resource use (Gemedo-Dalle et al., 2005). Targeted based studies with concentration on mechanism of action, effective dose and bioavailability mechanisms need to be conducted in future to explore medicinal potential of these plants so that the ill ethnic groups get maximum benefits from traditional medicinal system. Such scientific investigations are the baseline information for appropriate use of native medicinal plant species for drug development to cure various diseases (Cox and Balick, 1996; Flaster, 1996). Numerous studies on ethnomedicinal plants of Nepal have been conducted in the past by the Nepalese and foreign researcher. In the present study, some plant species used by three ethnic groups of Parbat to cure various human diseases are enumerated. As such, the present study was carried out to fill the gap of knowledge in this field of vital importance.

MATERIALS AND METHODS

This study was undertaken during 2011and 2012 in the different localities of Parbat to survey the information on ethnomedicinal uses of plants growing in this region (Figure 1). Regular monthly

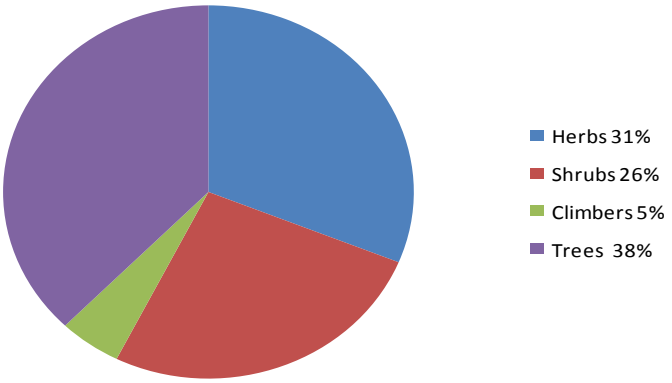

Figure 2. Percentage distribution of plants species according to habit.

visits were made to eleven different Village Developmental Committees (VDCs) of Parbat district for collecting ethnomedicinal information in particular. In this context, herbalist, senior knowledgeable men and women were interviewed for recording the ethnobotanical data. Local names and medicinal uses were documented critically. The herbarium specimens were made accordingly (Jain and Rao, 1997) and identified with the help of Flora of Nepal (Hara, 1966). Voucher specimens were deposited at the Department of Environmental Science and Engineering, Kathmandu University, Dhulikhel, Nepal.

Study area

Parbat, a hilly district situated in the Dhaulagiri zone of Western Nepal. It lies between 27° 58' N to 28° 39' N latitudes and 83° 34' E to 83° 59' E longitudes geographical limits in 536.86 km^2 area at altitudinal variation from 520 to 3309 m. Headquarter of the district is Kushma. The political boundaries of the district extend in the east to Syanga and Kaski districts; Baglung, Myagdi and Gulmi districts in the west; Myagdi and Kaski districts in the north and Syanja and Gulmi districts in the south (Figure 1). It has sub-tropical, temperate and sub-alpine monsoon type of climate with maximum temperature beyond 35°C during summer (May to June) and below 7.0°C during winter (December to January) and the northern part is moister, due to high rainfall and southern part is dry due to low rainfall. The average annual rainfall is 1950 ml. The major caste of the district is Brahmin (38.48%) followed by Chhetri (16.01%) and other major tribes inhabiting in this area are Gurung, Magar, Newar, Thakali, Kumal, Majhi, Bote, Puri, Snayasi, Tolange, Biswakarma, Pariyar, etc. For the present study only three ethnic groups are investigated, namely, Gurung, Magar and Majhi. In Parbat district, beside agriculture, animal husbandry is the main occupation of the tribal communities due to excess availability of fodders. Goats, cows, bulls, buffaloes and sheep are the main cattle of the local people in the district. Geographically, forest is divided into conifer forest (10.5%), broad leaved forest (69.5%), shrub forest (7%) and mixed forest (13%). It is traversed by two big rivers Kaligandaki and Modi as well as other rivulets, streams and streamlets, such as, Lungdi khola, Panyu khola, Luwa khola, etc.

RESULTS AND DISCUSSION

The first hand information on the ethnomedicinal plants used by the three ethnic tribes; Gurung, Magar and Majhi were arranged alphabetically by genus and species name (Table 1). A total of 61 plant species belonging to 59 genera in 43 families have been found to be used by the ethnic tribes for curing various human diseases (Plates 1 and 2). Out of the sixty one plant species, 31% were herbs, 26% were shrubs, 38% were trees and 5% were climbers (Figure 2).

Based on the surveyed ethnomedicinal data, a total of 61 plant species belonging to 59 genera under 43 families were used by different ethnic groups, the Gurung, Magar and Majhi of Parbat district. The present exploration provides ample information to believe that traditional medicinal practice of using the native medicinal plant is shown in the area. The traditional knowledge of the tribal people of Parbat district has tremendous ethnobotanical and ethnomedicinal importance. They commonly use plants and their parts such as roots, rhizomes, tubers, leaves, stem, wood, bark, flowers, seeds, and fruits in various purposes in their daily life. Several interesting observations were made during the course of the survey. Some of the plants used by the tribe have already been reported to have medicinal values. Furthermore, the uses of some plants are same as used by certain other ethnic groups of Nepal (Mahato and Chaudhary, 2005; Kunwar et al., 2006; Kunwar et al. 2010; Bhattarai et al., 2010). It is evident from the present study that the tribal communities are dependent on a variety of plants to meet their requirements and beliefs to cure various diseases. The different plant parts are used for medicinal preparation, mode of administration, dosage and other human consumption. In some cases, the whole plant parts are utilized only for medicinal purposes. Study revealed that a total of 47 human diseases are cured using different plant parts by the tribal people in Parbat district. The plant parts are generally used to cure some important diseases, namely, diarrhoea, dysentery, asthma, fever, stomach disorder, cuts and wounds, sore throat, rheumatism, blood pressure, urinary problems, ear diseases, headache, cough and cold, hypertension, venereal disease, scorpion bites, paralysis, blood pressure, diabetes, eczema, bone fracture, constipation and piles. The elder ethnic people are more familiar with the plant species and their use for common ailments, and the plant remedies being used regularly. Majorities of young generation do not know many plants and their medicinal values. Only a few younger ones followed the medicinal practices and traditional knowledge by the elders and healers as in the other areas of Nepal (Joshi and Edington, 1990; Shrestha and Dhillion, 2003; Malla and Chhetri, 2012). Scientific cultivation, conservation and sustainable use of plant species by ethnic communities would be highly advantageous for socioeconomic growth, in conservation of rare and endangered plant species and the indigenous knowledge for the future generations. Reported medicinal plants are

Plate 1. Plants recorded from different areas in Parbat district. (A) *Acorus calamus* L., (B) *Justicia adhatoda* L., (C) *Ageratum conyzoides* L., (D) *Girardinia diversifolia* (Link.) Friis, (E) *Berberis aristata* DC., (F) *Rhus javanica* L., (G) *Smilax ovalifolia* Roxb. Ex. D. Don., (H) *Schima wallichii* (DC.) Korth., (I) *Diploknema butyracea* (Roxb.) Lam, (J) *Woodfordia fructicosa* (L.) Kurz., (K) *Mucuna monosperma* (Roxb.) DC, (L) *Jatropha curcas* L., (M) *Berberis asiatica* Roxb. ex DC, (N) *Rubus ellipticus* Sm., (O) *Reinwardtia indica* Dum.

Plate 2. Plants recorded from different areas in Parbat district. (A) Gurung healer collecting *Swertia chirayita* (Roxb. ex Flem.) Karst., (B) *Osbeckia stellata* Buch.-Ham., (C) *Bauhinia variegate* L., (D) *Rubia manjith* Roxb. ex Flem., (E) *Zanthoxylum armatum* DC, (F) *Phyllanthus emblica* L., (G) *Englehardtia spicata* Lesch. ex.Blume, (H) *Choerospondias axillaris* (Roxb.) Burt & Hill, (I) A woman with *Achyranthes bidentata* Blume., (J) *Juglans regia* L., (K) *Cuscuta reflexa* Roxb., (L) *Cleistocalyx operculatus* Roxb. Murr. & Perry., (M) Grinding bark of *Cleistocalyx operculatus* Roxb. Murr. & Perry, (N) *Cinnamomum tamala* (Buch.-Ham.) Nees & Eberm., (O) *Bombax ceiba* L.

strongly recommended for further nutritional, phytochemical and pharmacological studies.

Conclusion

This study showed that the study area has ethnomedicinal plants used to treat wide variety of human ailments. According to the tribal communities and their traditional practices, majority of the ethomedicinal plants are selected for the treatment of human ailments. The tribal people used medicinal plants in the treatment of some very common diseases such as indigestion, diarrhoea and dysentery, muscular swelling, fever, scabies, ear

Table 1. Information on ethnomedicinal plants used by ethnic tribes of Parbat district Nepal.

S/N	Botanical name/Voucher number	Family	Local name	Locality	Plant parts used and mode of application
1	*Achyranthes bidentata* Blume. Malla-0368	*Amaranthaceae*	Datiwan (N)	Lunkhu Deurali	Root juice is recommended for hypertension and rheumatism. Paste of root is used for toothache.
2	*Acmella calva* (Candolle) R.K Jansen Malla-0386	Asteraceae	Marethi (N)	Bajung	The fruit juice is given for stomach disorder. It is also used in fish poisoning.
3	*Acorus calamus* L. Malla-0392	Araceae	Bojho (N, G, M)	Lamtun	Rhizomes are chewed to get relief from sore throat and voice disorder. Decoction of rhizomes is taken to treat cold, fever, diarrhoea. Powder of rhizome is put in a cup of tea for flavor.
4	*Ageratum conyzoides* L. Malla-0353	Asterceae	Ganaune jhar (N)	Bhorle	Whole plant juice is applied to blood clotting in fresh cuts and wounds.
5	*Aloe vera* (L.) Burm. f. Malla-0358	Liliaceae	Ghyukumari (N, M), Chhigu (G)	Sarthan	The gel of leaves is used to relief from burning sensation. Juice is used to treat pain, fever, constipation and jaundice.
6	*Amaranthus spinosus* L. Malla-0380	Amaranthaceae	Lundekanda (N), Chikli, Lode (G), Lunde (M), Gandri (Maj)	Lunkhu Deurali	Root juice is administrated for fever and urinary trouble. A paste of the root is applied to treat gonorrhea. Fresh leaves and tender shoots are consumed as a vegetable.
7	*Amomum subulatum* Roxb. Malla-0403	Zingiberaceae	Alainchi (N)	Simle	Seeds are chewed raw for indigestion and to stop vomiting. Paste of the seed is used as falvour.
8	*Artemisia indica* Willd. Malla-0435	Asteraceae	Titepati (N), Chyonre, Pacha (G), Pati (M)	Phalamkhani	Leaves and young shoots are applied to cure bleeding of wounds and nose, asthma, fever, headache and diarrhoea.
9	*Artocarpus lakoocha* Wall. ex Roxb. Malla-0388	Moraceae	Badahar (N)	Sarthan	Bark juice is applied to cuts and wounds. Ripen fruits are eaten fresh and immature ones are cooked as a curry.

Table 1. Contd.

10	*Azadirachta indica* A. Juss. Malla-0431	Meliaceae	Neem (N,G, M)	Kusma	Barks and leaves are crushed in powder and made juice in boil water for the treatment of fever, intestinal worms, ulcers, liver and urinary complaints, cough, headache and body pain. Flowers are useful for piles and leprosy. Fruits juice is used for urinary disorder and skin diseases.
11	*Bauhinia variegata* L. Malla-0396	Caesalpiniaceae	Koiralo (N, Maj), Byahagan, Kurugan (M)	Kokhe	Bark juice is drunk for tonic and blood purifier. Bark paste is applied to cuts and wounds. Fresh flowers are cooked as vegetable and made pickle.
12	*Berberis aristata* DC. Malla-0410	Berberidaceae	Banchutro (N), Chutro (G)	Chitre	Root juice is used in fever and dysentery. Bark paste is used for piles, sore throat and skin disease. The ripen fruits are eaten. Barks are also the sources of yellow dye.
13	*B. asiatica* Roxb. ex DC. Malla-0426	Berberidaceae	Chutro (N, M), Chotr (G)	Phalamkhani	Bark juice is used for dysentery and piles. Fresh and ripe fruits are eaten and also used to distill alcohol.
14	*Bombax ceiba* L. Malla-0487	Bombacaceae	Simal (N, M, Maj), Chongonchhi (G)	Simle	Bark juice is useful for diarrhoea, dysentery and tuberculosis. Root juice is used in wounds. Flowers are good for skin problems. Fruits are also useful in treating gonorrhea and eaten.
15	*Cannabis sativa* L. Malla-0494	Cannabaceae	Gaanja, Ganja (N,G) Bhango (M)	Simle	Dried powder of leaves or flowers is drunk with milk to cure cough, asthma, diarrhoea and dysentery. Seeds are roasted and pickled. Dried leaves are mixed with tobacco and used for smoking. It is also offered to god Shiva in rituals.
16	*Centella asiatica* (L.) Urb. Malla-0412	Apiaceae	Ghodtapre (N, M), Jasundo, Topre jhar (G)	Lunkhu Deurali	Leaves are useful for remedy of skin diseases. Whole plant parts are ground and juice about four teaspoons six times a day is used to treat fever and indigestion.
17	*Cheilanthes dalhousiae* Hook Malla-0434	Pterridaceae	Ranisinka (N, M)	Patichaur	Whole plant juice is used to cure ulcer, stomach ache. Stem is used to thread children's ear.

Table 1. Contd.

18	*Choerospondias axillaris* (Roxb.) Burt & Hill Malla-0416	Anacardiaceae	Lapsee, Labasi (N), Khaiya, Kalan (G)	Durlung	Fruits are eaten fresh or pickled. Farmers normally process the fruits for their household needs as pickles and chutney, etc. by crushing and boiling the fruits, whereas entrepreneurs purchase the fruits from growers and produce varieties of edible pulp cake indigenously called Titaura items for selling in the market of Nepal.
19	*Cinnamomum tamala* (Buch.-Ham.) Nees & Eberm. Malla-0377	Lauraceae	Tejpat (N), Lepe (G)	Sarthan	Bark juice is useful for diarrhoea and nausea. Leaves are used as stimulant in tea and making foods and meat. It is also used as spices.
20	*Cleistocalyx operculatus* Roxb. Murr. & Perry Malla- 0391	Myrtaceae	Kyamuno (N), Kemna (G), Kyamuna (M)	Sarthan	Bark is crushed and made juice to cure muscular swellings caused by external injury of cattle's. Barks and young leaves are used to make alcoholic beverage. Leaf juice is used to treat throat problems. Fresh ripe fruits are eaten. Powder of leaves is smoked in case of cold.
21	*Crataeva unilocularis* Buch.-Ham. Malla-0398	Capparidaceae	Sipleegaan (N)	Simle	Leaves paste is used to cure rheumatism. Bark juice is taken to cure kidney problems. Juice of the young leave is taken to cure headache. Young twigs are taken as a vegetable.
22	*Cuscuta reflexa* Roxb. Malla-0405	Convolvulaceae	Akasebeli (N), Dyo dyoali (G)	Phalamkhani	Plant juice doses of about four teaspoons twice a day is used to treat jaundice and fever.
23	*Cynodon dactylon* L. Pers. Malla-0375	Poaceae	Dubo (N, M), Nodubo (G)	Kokhe	Plant juice of about eight teaspoon is used to cure indigestion. Paste of the plant is used for the treatment of bleeding from nose and wounds. Leaves are used in many rituals.
24	*Dendrophthoe falcata* (L.) f. Etting. Malla-0414	Loranthaceae	Aijeru (N)	Bajung	Bark juice is used for treating asthma, tuberculosis, menstrual disorders and swelling. Paste of the fruits is applied to set dislocated bones.
25	*Diploknema butyracea* (Roxb.) Lam Malla-0363	Sapotaceae	Chyauree, Chyuri (N, M),	Sarthan	Ripen fruits are taken as sources of vitamin. Bark juice about four teaspoon is given to cure indigestion. Fruits are used to make alcoholic beverage. Flowers are very useful for bees to make honey and leaves are looped for fodder.

Table 1. Contd.

26	*Englehardtia spicata* Lesch. Ex Blume Malla-0365	Juglandaceae	Mahuwa (N)	Bhorle	Bark juice is given to cure sore throat and bronchitis. Yong leaves are mixed with sand and ground and added to water body for fish poisoning.
27	*Eupatorium odoratum* L. Malla-0361	Asteraceae	Banmara (N), Besi banmara (M), Thulo banmara (Maj)	Chitre	Decoction of the plant is used for removing lice and fish poisoning.
28	*Euphorbia royleana* Boiss. Malla-0356	Euphorbiaceae	Siundee (N), Syuri (G), Dha (M), Kanpate (Maj)	Chuwa	Leaf latex is used to cure fever, mumps and cuts. Latex is warmed over a fire and applied to wounds between the toes especially during rainy season while walking on muddy water. It is also used for fish poisoning.
29	*Ficus lacor* (Buch.)-Ham. Malla-0354	Moraceae	Kabhro (N), Kapara (M)	Sarthan	Bark juice is used to treat ulcers. Seeds are useful in scabies. Flowers are pickled. Bark gives fiber that is used for rope. Plant is looped as fodder.
30	*Fraxinus floribunda* Wall. Malla-0376	Oleaceae	Lankuri (N), Raunle (G)	Simle	Stem juice and resin are used to treat stomach disorder in sheep and goats. Wood is used to make agricultural equipments and tools (‘Juwa’ in Nepali). Bark paste is used for the treatment of broken legs and arms.
31	*Girardinia diversifolia* (Link.) Friis Malla-0433	Urticaceae	Chalne sisno (N)	Chitre	Juice of the roots can be used for treating gastritis, constipation and diabetes, while juice of the leaves is used for head ache, fever, joint aches and tuberculosis. However, more treatment except boiling.
32	*Jatropha curcas* L. Malla-0401	Euphorbiaceae	Sajiwan (N), Rajani giri, Sajin (G), Ratyun, Aren (Maj)	Ranipani	The plant juice is used in syphilis and pneumonia. Roots are ground and applied in rheumatism and dysentery. Young stem is used to wash teeth.
33	*Juglans regia* L. Malla-0420	Juglandaceae	Okhar (N), Akhor, Katu (G), Okhar (M)	Lunkhu Deurali	Leaf juice is used as tonic. Bark and fruits are used for detergent.
34	*Justicia adhatoda* L. Malla-0357	Acanthaceae	Asuro (N), Aasuri (G)	Sarthan	Leaves are boiled in water, filtered and used to cure cough, bronchitis and asthma. It is administered to cure fever, about two teaspoons three times a day. Leaves are used as green compost on the paddy field.

Table 1. Contd.

35	*Lindera neesiana* (Wall. ex Nees) Kurz. Malla-0418	Lauraceae	Siltimur (N, M), Gutum (G)	Karkineta	The fruits are chewed to cure diarrhoea and toothache. Leaves are used to cure skin diseases. Fruit juice is used to treat stomach disorder of livestock if they eat poisonous plants.
36	*Macrotyloma uniflorum* Lam. Verdc. Malla-0411	Papilionaceae	Gahat (N)	Ranipani	Seeds are cooked and eaten. Seeds soup is used to treat menstrual disorders. Soup is also useful for body pain and used for curing the stone of kidney.
37	*Mahonia napaulensis* DC Malla-0421	Berberidaceae	Jamanemandro (N), Komo (G)	Chitre	The bark juice is taken to cure dysentery, diarrhoea. Ripe fresh fruits are eaten and pickled. Flowers are used as ornamental purpose.
38	*Mucuna monosperma* (Roxb.) DC Malla-0351	Leguminosae	Baldhangro (N), Kaucho (M)	Bhorle	Bark juice is used for fever and bristle-poisonous. Seeds are used for detergent.
39	*Nephrolepsis cordifolia* (L) C. Presl. Malla-0417	Neprolepidaceae	Pani amala (N)	Phalamkhani	A decoction of the plant is used for cooling cough. Tubers are eaten fresh for shore throat.
40	*Nicotiana tabacum* L. Malla-0419	Solanaceae	Surtee (N) , Tamahun (G), Bhusa (M)	Durlung	Leaf past is used to treat rheumatic swellings, skin diseases and scorpion sting. Juice of leaves is used to cure cuts, wounds and removal of cattle lice.
41	*Ocimum sanctum* L. Malla-0381	Lamiaceae	Tulsipat (N)	Ranipani	The juice of leaves is used to cure fever, stomachic, bronchitis and earache. Leaves are directly eaten for gastric disorder. It has also used for spiritual purpose.
42	*Osbeckia stellata* Buch.-Ham. Malla-0413	Melastomataceae	Angaru (M), Paglya jhar (G)	Deupur	Juice of the root about 3 teaspoons twice a day is given to treat diarrhoea and dysentery. A decoction of the plant is also given to domestic animals when they eat poisonous plants. Juice of the leaves is applied to treat scabies. Ripe fruits are eaten fresh.

Table 1. Contd.

43	*Oxalis corniculata* L. Malla-0390	Oxalidaceae	Chariamilo (N), Chino (G), Chari amilo (M), Chariumal (Maj)	Ranipani	Leaves are crushed to make juice to cure stomachic and fever. Whole plant is used for preparing ‘chutney’.
44	*Phyllanthus emblica* L. Malla-0385	Euphorbiaceae	Amala (N, Maj), Kyun, Titi (G), Aaunlesa (M)	Ranipani	Roasted fruits are eaten to cure diarrhoea, dysentery, sore throat and prepared pickle. Bark juice is used for bronchitis.
45	*Prunus cerasoides* D. Don Malla-0404	Rosaceae	Paiyau (N), Chyarbu (G), Pange (M)	Deupur	Bark juice is used to cure swelling. Ripe fresh fruits are eaten. The plant is looped as fodder for livestock
46	*Reinwardtia indica* Dum. Malla-0366	Linaceae	Pyaulee (N), Gyumi (G), Gebatisar (M)	Lamtun	Past of root is applied to headache. The juice of the root is given to treat fever, scabies, wounds and indigestion.
57	*Rhododendron arboretum* Sm. Malla-0432	Ericaceae	Laleegurans (N), Porota, Pota (G), Lalisar (M)	Chitre	Juice of bark is used for treatment of cough, dysentery and diarrhoea. The petals are eaten raw to get relief from menstrual disorders and are chewed in case of a fish bone is stuck in the neck. Petals are used to prepare alcoholic beverage.
48	*Rhus javanica* L. Malla-0362	Anacardiaceae	Vakiamilo (N), Ghursing (G), Muruk (Mag.)	Bhorle	Fruits are ground to make juice and used to treat diarrhoea, blood dysentery and paralysis. A past of the fruits is applied to treat swelling and wounds. Fruits are chewed in case of stomachache and as appetizer.
49	*Rubia manjith* Roxb. ex Flem. Malla-0402	Rubiacese	Majitho (N), Tiro lahara (M)	Deupur	Juice of the leaves about 4 teaspoons three times a day is given to treat urinary problem and diabetes. A paste of the stem is applied to scorpion bites. The root is used for reddish dye.
50	*Rubus ellipticus* Sm. Malla-0374	Rosaceae	Ainselu (N), Melanchi, Palan (G), Dhewasi (M), Jyaunsi (Maj)	Chitre	Leaves are ground and taken for relief gastric disorder. The fresh ripe fruits are eaten and sold in the market.
51	*Sapindus mukorossi* Gaertn. Malla-0370	Sapindaceae	Reetha (N), Jharlyang (M)	Ranipani	Seeds are ground and used as detergent to washing cloths and hair. The lather of the fruits is used to treat burns.

Table 1. Contd.

52	*Sapium insigne* (Royal) Benth. Ex Hook. Malla-0352	Euphorbiaceae	Khirro (N), Mibalang (M), Khirra (Maj)	Lamtun	Milky latex of the plant is used around navel of child to cure diarrhoea. It is also used to remove worms and germs of the wounds. Leaves juice is used for fish poison.
53	*Schima wallichii* (DC.) Korth. Malla-0364	Theaceae	Chilaune (N), Kyosin (G), Hyansing (M)	Simle	The powder of bark is used to cure cuts and liver flukes in animals. Young leaves and bark are paste is used for fishing by local people. Wood is used as timber for construction.
54	*Smilax ovalifolia* Roxb.ex.D.Don Malla-0395	Liliaceae	Kukurdaino (N)	Phalamkhani	Root juice is used to cure venereal disease, rheumatism and wounds.
55	*Swertia chirayita* (Roxb. ex Flem.) Karst. Malla-0408	Gentianaceae	Chiraito (N), Tento (G), Rauka (M)	Chitre	The leaves juice is excellent drug for fever, skin diseases, intestinal worms, asthma and diarrhoea. The paste of the plants is applied to cure eczema.
56	*Syzygium cuminii* (L.) Skeels. Malla-0372	Myrtaceae	Jamun (N), Jamuna (G, M), Phandil (Maj)	Bhorle	Bark is crushed and used to cure sore throat, asthma, dysentery and diarrhoea. The ripen frits are eaten. Timber is used for home applicants.
57	*Terminalia bellirica* (Gaertn.) Roxb. Malla-0359	Combretaceae	Barro (N, G)	Phalamkhani	Roasted seeds are chewed for treating sore throat, stomach disorders, indigestion, and piles. Leaves are lopped for palatable fodder.
58	*T. chebula* Retz. Malla-0383	Combretaceae	Harro (N)	Lunkhu Deurali	Dried bark is chewed to treat inflammation of tonsils. Roasted fruits are used for the remedy of piles and cold. Leaves are looped for fodder.
59	*Urtica dioica* L. Malla-0378	Urticaceae	Sisnu (N), Polo, Pulu (G), Dhyo, Hyo (M)	Simle	Root juice is administered for asthma, blood pressure and sugar. The Magars of Parbat district add sparrow and rat droppings to paste of the root to treat cuts and wounds. Tender leaves and inflorescences are cooked as vegetables and taken to lower hypertension.
60	*Woodfordia fruticosa* (L.) Kurz. Malla-0399	Lythraceae	Dhairo (N), Dhanyar (G), Dhainra (M), Dhauli (Maj)	Bhorle	Dried powder of flowers is used to cure dysentery and diarrhoea. The dried flowers are mixed with ‘Marcha’ yeast to make alcoholic beverage (Gurung, Magar). Leaves are lopped as palatable fodder to goats.

Table 1. Contd.

61	*Zanthoxylum armatum* DC. Malla-0422	Rutaceae	Timur (N, M, Maj), Prumo (G)	Phalamkhani	The bark is ground and taken for relief fever, cholera and stomach disorder. Fruits are chewed in toothache and stomachic. Fruits are widely used for making pickles. Fruits and thorns are used for fish poisoning (Majhi).

Nepali (N), Gurung (G), Magar (M), and Majhi (Maj).

ache, cuts and wounds, peptic ulcer and backache. Thus, it is necessary to acquire and preserves this traditional system of medicine by proper documentation and identification of plant species. This traditional knowledge on the indigenous uses of the medicinal plants could boost new innovations in the pharmaceutical industries and have many beneficial applications such as new medicinal trails for some pernicious diseases like cancer and AIDS.

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Evaluation of the antioxidant and antiradical activities of perilla seed, leaf and stalk extracts

En-Shyh Lin[1]*, Chia-Ching Li[2] and Hung-Ju Chou[2]

[1]Department of Beauty Science, National Taichung University of Science and Technology, Taichung 40343, Taiwan.
[2]Department of Cosmetic Science, Vanung University, Taoyuan 32061, Taiwan.

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The antioxidant properties of perilla seed, leaf and stalk extracts were evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide radicals scavenging activities, reducing power and metal chelating ability. The DPPH radical scavenging activity of perilla leaf extract was 57.5%, a value which was higher than scavenging activity of other perilla extracts at 15.0 µg/ml. Based on superoxide radical scavenging effect, the perilla leaf extract was the most effective (64.2%) one followed by perilla seed extract (55.1%) and perilla stalk extract (20.1%) at 10.0 µg/ml. Reducing powers of perilla seed, leaf and stalk extracts were excellent. Chelating effects of perilla seed, leaf and stalk extracts were increased with the increasing concentration. The data shows that perilla leaf extract has effective antioxidant functions, especially in DPPH and superoxide radicals scavenging activities and reducing power. The results indicate that the 50% aqueous-methanol extracts of perilla leaf can be used as a new functional food and pharmaceutical agent.

Key words: Perilla, scavenging activity, reducing power, chelating effect, phenolics compound.

INTRODUCTION

Free radicals are a major interest for physiological and biochemical lesions. Although oxygen is essential for aerobic forms of life, oxygen metabolites are highly toxic. As a consequence, reactive oxygen species (ROS) are known to be implicated in many cell disorders and in the development of many diseases including coronary artery disease, aging and cancer (Gülçin 2010). Antioxidants inhibit or prevent oxidation of substrates and evolve to protect biological systems against damage induced by ROS. Interest in finding naturally occurring antioxidants in foods or medicines to replace synthetic antioxidants has increased considerably, given that synthetic antioxidants are being restricted due to their side effects (Zheng and Wang, 2001). Therefore, interest in finding natural antioxidants, without undesirable side effects, has increased greatly. The numbers of antioxidant compound by plants play important roles in preventing diseases induced by free radicals (Hirose et al., 1994). Therefore, attention has been directed toward the development of natural antioxidants from plant sources (Chou et al., 2009; Lin et al., 2010b; Lin and Li, 2010).

Perilla (*Perilla frutescens*, Labiatae) is one of popular traditional herbal medicines used in Taiwan and widely distributed in China, Japan and Korea. In general, perilla is used individually in Chinese medicine to treat a variety of diseases (Chinese Pharmacopoeia Commission, 2005). The seeds are employed for dyspnea and cough relief, phlegm elimination and the bowel relaxation. The leaves are said to be helpful for asthma, cold and flu, and to regulate stomach function, while the stalk of the plant is traditionally used as an analgesic and anti-abortive agent (Chinese Pharmacopoeia Commission, 2005). Previously, some studies were focused on antioxidant activity of *Perilla pankinensis* extract (Gülçin et al., 2005)

*Corresponding author. E-mail: eslin7620@hotmail.com. Tel: +886-4-22196968. Fax: +886-4-22195891.

and *Perilla frutescens* extract (Chou et al., 2009; Kim et al., 2007; Kim et al., 2008; Lin et al., 2010a). In addition, various medicinal properties of perilla have been ascribed to the antioxidant, anti-inflammatory, anti-allergic and anti-tumor promoting substances (Lin et al., 2007; Makino et al., 2003; Takano et al., 2004; Ueda et al., 2002; Žekonis et al., 2008).

The extraction method was a major factor to determine the composition and their effective in the plant extract. The aims of this work were to estimate the phenolic content and to evaluate the antioxidant and antiradical activities of 50% aqueous-methanol extracts of perilla seed, leaf and stalk. Their antioxidant and antiradical activities were also compared to well known natural antioxidants, such as ascorbic acid and gallic acid.

MATERIALS AND METHODS

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), phenazine methosulfate (PMS), nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium (NBT), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), gallic acid (GA) and ethylenediamine-tetraacetic acid (EDTA) were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). Ascorbic acid (Vit C) was purchased from Fluka (Buchs, Switzerland). All other reagents were of analytical grade.

Extraction procedure

The perilla seed, leaf and stalk were purchased locally (Goangder Tarng Ginseng Co., Taoyuan, Taiwan). The areca flower extracts were prepared according to Köksal and Gülçin (2008) with modification (Chou et al., 2009). The dried of perilla seed, leaf and stalk were ground in a mortar, and extracted twice that combined the same volume of distilled water and methanol under reflux for 4 h at 70°C. The supernatant was separated from the solid residue by paper filtration (No. 1, Advantec, Tokyo, Japan). The 50% aqueous-methanol extracts were combined and evaporated at 60°C under reduced pressure. All dried extracts were stored at 4°C until use.

Total phenolic determination

Total phenolic contents were quantified according to the method of Chou et al. (2009). Basically, aqueous-methanol extract solution (50 µl) was mixed with 50 µl of Folin-Ciocalteu's phenol reagent. Then, 500 µl of a 15% sodium carbonate solution was added to the mixture and it was adjusted to 400 µl with distilled water. The mixture was allowed to stand for 10 min with intermittent shaking, after which the absorbance was read at 725 nm (Ultrospec 2100 pro spectrophotometer, Amersham Pharmacia Biotech, UK). Results were expressed as gallic acid equivalent. All experiments were done in triplicate.

DPPH radical scavenging activity

The DPPH radical scavenging activity was measured according to the method of Chou et al. (2009). A stock solution of DPPH radical (1 mM) in methanol was prepared. An aliquot (100 µl) of 1 mM methanol solution of DPPH was mixed with varying concentrations of the perilla extract. After a 30 min incubation period at room temperature, the absorbance of the resulting solution was read at 517 nm. For each sample a methanol blank was also measured. DPPH radical scavenging activity was expressed as the inhibition percentage and was calculated as (1 – absorbance of sample/ absorbance of control) × 100. The IC_{50} value (µg/ml) is the effective concentration at which the DPPH radicals were scavenged by 50% and was obtained by interpolation from linear regression analysis. Ascorbic acid and gallic acid were used for comparison.

Superoxide radical scavenging effect

Scavenging activity on superoxide radical was evaluated according to the method of Chou et al. (2009). The reaction mixture contained the same volume of 120 µM PMS, 936 µM NADH, perilla extract, and 300 µM NBT in a total volume of 1 ml of phosphate buffer (100 mM, pH 7.4). After 5 min of incubation at ambient temperature, the absorbance of the resulting solution was measured at 560 nm. The superoxide radical activity was calculated as scavenging effect (%) = (1 – absorbance of sample/absorbance of control) × 100. The IC_{50} value (µg/ml) is the effective concentration at which the superoxide radical were scavenged by 50%. Ascorbic acid and gallic acid were used for comparison.

Reducing power

The reducing power was determined according to the method of Chou et al. (2009). The perilla extract (250 µl) was mixed with 250 µl of 200 mM sodium phosphate buffer (pH 6.6) and 250 µl of 1% potassium ferricyanide, and the mixture was incubated at 50°C for 20 min. Later, 250 µl of 10% trichloroacetic acid was added to the mixture to stop the reaction. The mixture was centrifuged at 3000 rpm for 10 min. The supernatant (500 µl) was mixed with 400 µl of deionized water and 100 µl of 0.1% ferric chloride solution, allowed to stand for 10 min, and the absorbance was measured at 700 nm. A higher absorbance indicated a higher reducing power. The IC_{50} value (µg/ml) is the effective concentration at which the absorbance was 1.0 for the reducing power. Ascorbic acid and gallic acid were used for comparison.

Chelating effect on ferrous ions

The chelating effect of ferrous ions was estimated according to the method of Chou et al. (2009). The perilla extract was added to a solution of 2 mM $FeCl_2$ (20 µl). The reaction was initiated by the addition of 5 mM ferrozine (40 µl) and the mixture was shaken vigorously and left standing at room temperature for 10 min. The absorbance of the mixture was measured at 562 nm. Chelating effect was calculated using the equation: (1 – absorbance of sample/absorbance of control) × 100. The IC_{50} value (µg/ml) is the effective concentration at which ferrous ions were chelated by 50%. EDTA and citric acid were used for comparison. The data were presented as the means ± standard deviation (SD) of triplicate parallel measurements. Statistical analysis was performed using Student's t-test and $p < 0.05$ was regarded as significant.

RESULTS

We measured the total phenolic content to investigate if they may contribute to the antioxidant activity of the perilla extracts. As shown in Table 1, the total phenolic contents in perilla seed, leaf and stalk extracts were 424.86 ± 4.68, 549.00 ± 9.97 and 586.69 ± 2.74 µg/ml

Table 1. Total phenolic content of perilla seed, leaf and stalk extracts.

Extract	Seed	Leaf	Stalk
Total phenolic content[a] (μg/ml)	424.86±4.68	549.00±9.97	586.69±2.74

[a]Values represented mean ± SD of three parallel measurements. Total phenolic content was expressed as μg gallic acid equivalents/ml extract.

Table 2. IC_{50} values of perilla seed, leaf and stalk extracts and standard in antioxidant properties.

Parameter	Samples (μg/ml)					
	Seed	Leaf	Stalk	Vit. C	GA	EDTA
DPPH radical scavenging activity	16.52	10.89	11.04	10.79	3.59	nd[a]
Superoxide radical scavenging effect	5.96	6.93	16.11	8.39	10.48	nd[a]
Reducing power	21.46	16.38	15.80	12.27	12.32	nd[a]
Chelating ability on ferrous ions	62.78	71.27	72.39	nd[a]	nd[a]	8.82

[a]nd: not detected.

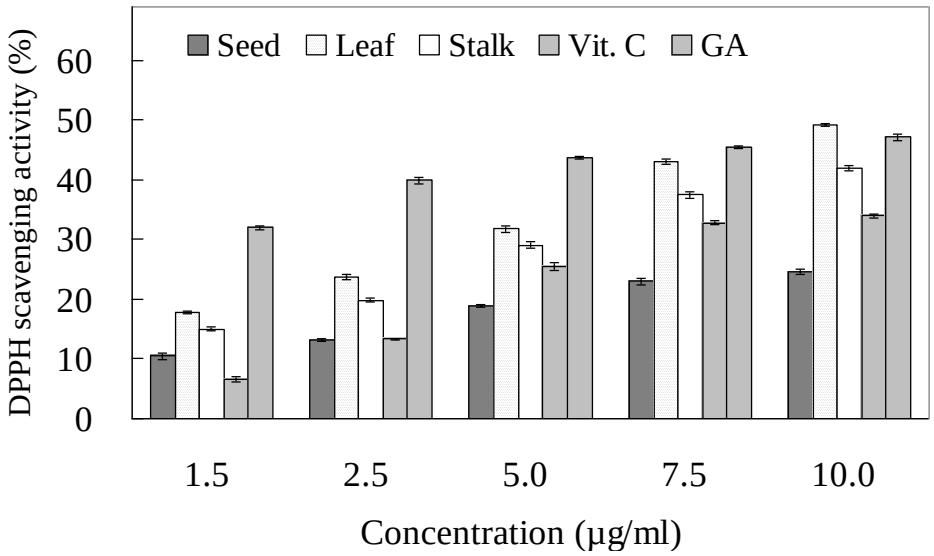

Figure 1. DPPH radical scavenging activity on of perilla seed, leaf and stalk extracts. Ascorbic acid and gallic acid were taken as the standards. Data expressed as mean ± SD (*n*=3).

extract, respectively. Obviously, the phenolic content of perilla stalk extract was higher than those of perilla leaf and perilla seed extracts. The results are expressed as gallic acid equivalents. The free radical scavenging activities of perilla extracts, measured by the DPPH method, are shown in Figure 1. The extracts of perilla leaf and perilla stalk had better DPPH radical scavenging activity than perilla seed extract at the same concentration. The perilla extract concentrations were expressed as gallic acid equivalent/ml extract. The scavenging effects of perilla extracts and standards on the DPPH radical were in the following order: gallic acid > perilla leaf > perilla stalk > ascorbic acid = perilla seed (39.9, 23.8, 19.9, 13.3 and 13.3%, respectively), at the same concentration (2.5 μg/ml). The extracts of perilla leaf and perilla stalk showed significant dose-dependent scavenging activity, especially, and it reached up to 49.2 and 42.0%, respectively, at the concentration of 10.0 μg/ml. As shown in Table 2, the IC_{50} value for perilla seed, perilla leaf and perilla stalk extracts in this assay were 16.52, 10.89 and 11.04 μg/ml, respectively. On the other hand, IC_{50} values of ascorbic acid and gallic acid were found to be 10.79 and 3.59 μg/ml, respectively.

The superoxide radical scavenging activities of perilla

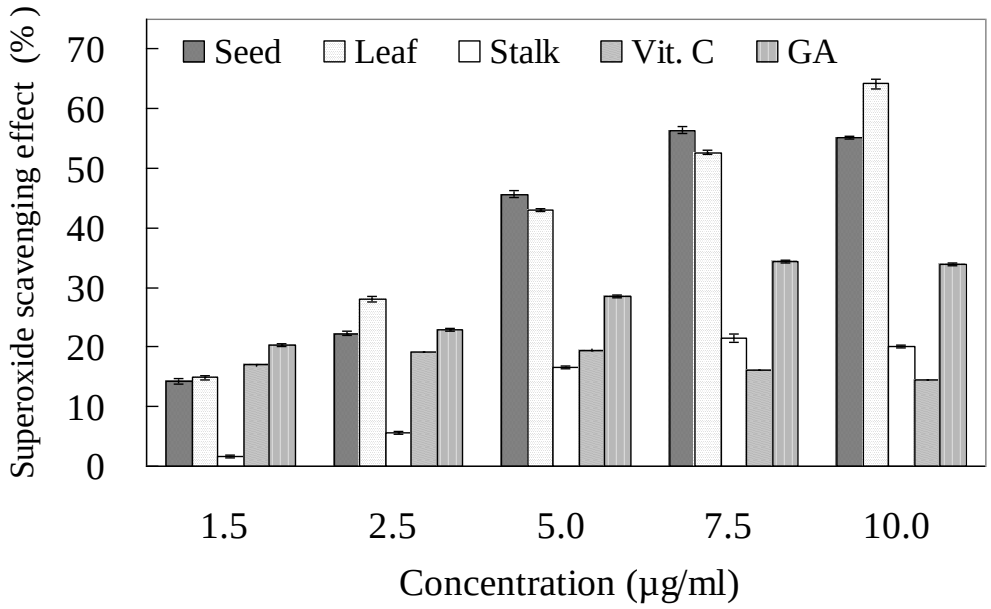

Figure 2. Superoxide radical scavenging effect of perilla seed, leaf and stalk extracts. Ascorbic acid and gallic acid were taken as the standards. Data expressed as mean ± SD (*n*=3).

extracts were increased with increasing sample concentrations (Figure 2). The extracts of perilla leaf and perilla seed showed significant dose-dependent scavenging activity on superoxide radical. Scavenging abilities of perilla leaf and perilla seed extracts were similar and better than that of perilla stalk; these activities were more than that of the standards at the concentration of 2.5 to 10.0 µg/ml. The extracts of perilla seed and perilla leaf exhibited good superoxide radical scavenging activity (IC_{50} = 5.96 and 6.93 µg/ml, respectively) that were comparable with standards (8.39 µg/ml for ascorbic acid and 10.48 µg/ml for gallic acid). The extract of perilla stalk had shown a very weak activity, IC_{50} = 16.11 µg/ml. All results are shown in Table 2.

The reducing power indicates compounds that are electron donors which can act as primary and secondary antioxidants (Yen and Chen, 1995). As seen in Figure 3, the reducing powers of perilla extracts were increased with increasing amount of sample, but values remained lower than that for ascorbic acid and gallic acid, at the concentration of 2.5 to 25.0 µg/ml. However, the reducing powers of perilla were similar in the following order: perilla stalk (2.02) > perilla leaf (1.99) > perilla seed (1.78) at the concentration of 37.5 µg/ml (ascorbic acid and gallic acid were 1.97 and 1.96, respectively). In addition, reducing powers of perilla extracts and standards were found similar statistically at 50.0 µg/ml (data not shown). The perilla leaf and perilla stalk extracts had shown higher reducing power with IC_{50} = 16.38 and 15.80 µg/ml, respectively. The IC_{50} for perilla seed was 21.46 µg/ml (Table 2). However, these values of ascorbic acid and gallic acid were found to be 12.27 and 12.32 µg/ml, respectively.

Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complexes decreases (Gülçin et al., 2009). Figure 4 shows that the absorbance of Fe^{2+}-ferrozine complex was decreased dose-dependently from 12.5 to 100 µg/ml for all perilla extracts. The iron chelating abilities of all perilla extracts showed moderate were in the range of 4.9 to 9.3% to 62.6 to 67.4%. Obviously, all perilla extracts had a steady increase on metal chelating activity with increasing concentrations. IC_{50} values of perilla seed extract was 62.78 µg/ml whereas that of perilla leaf and perilla stalk extracts were 71.27 and 72.39 µg/ml, respectively (Table 2). However, EDTA showed very strong chelating ability of 94.2% at 12.5 µg/ml (IC_{50} = 8.82 µg/ml) and citric acid had no iron ion chelating.

DISCUSSION

Antioxidant activity assays employed the inhibition of free radical DPPH test/method which is one of the oldest and most frequently used methods for total antioxidant potential/capacity of food and biological extracts. The radical scavenging activity values were expressed as the ratio percentage of sample absorbance decrease and the absorbance of DPPH solution in the absence of extract at 517 nm (Gülçin 2009). The decrease in absorption is taken as a measure of the extent of radical scavenging. From the analysis of Figure 1, the scavenging activities on DPPH radical of all perilla extracts increased with the concentration increase and were excellent, especially for perilla leaf extract. The DPPH radical scavenging activity

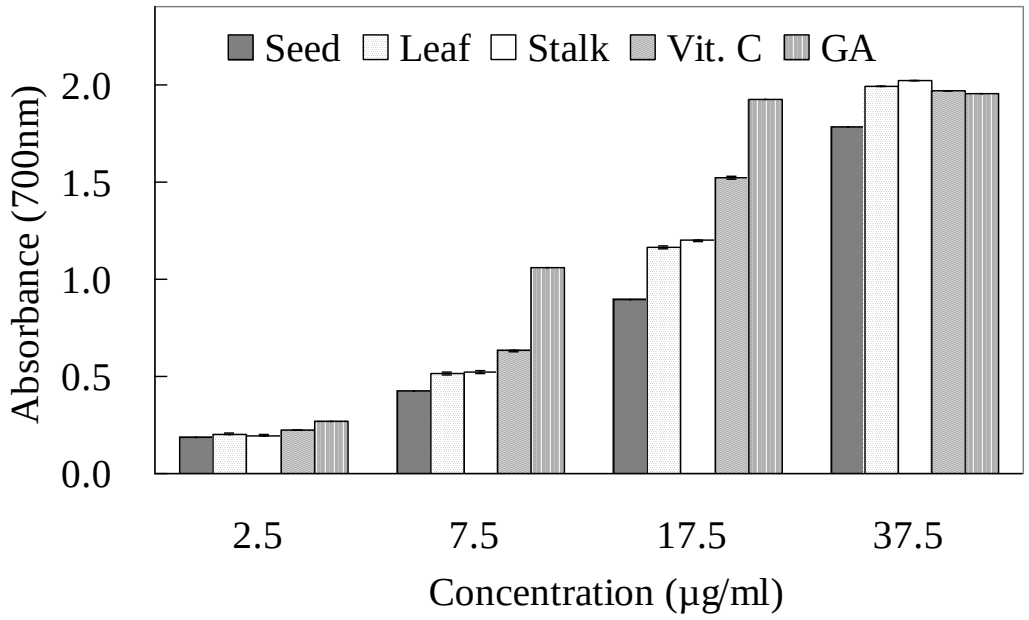

Figure 3. Reducing power of perilla seed, leaf and stalk extracts. Ascorbic acid and gallic acid were taken as the standards. Data expressed as mean ± SD (*n*=3).

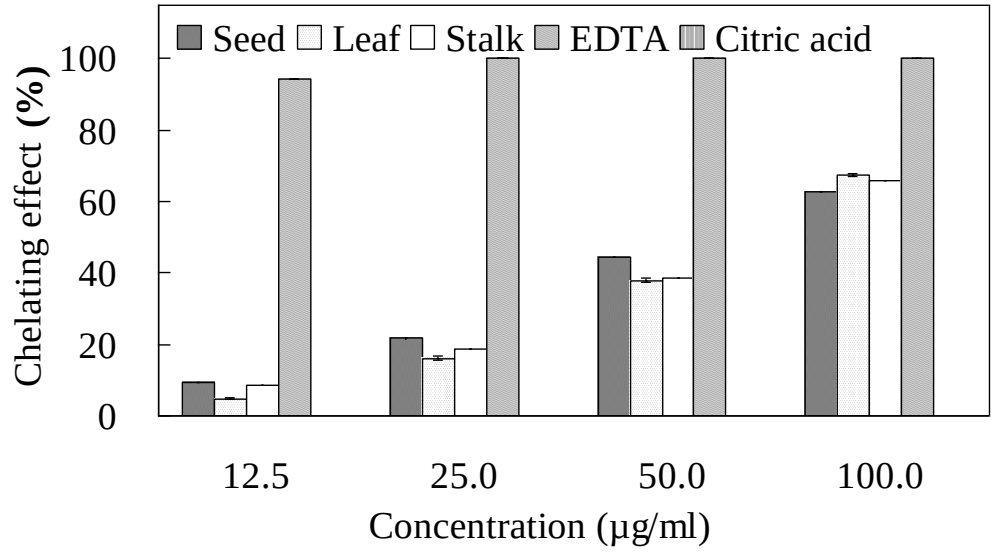

Figure 4. Chelating effect of perilla seed, leaf and stalk extracts on ferrous ions. EDTA and citric acid were taken as the standards. Data expressed as mean ± SD (*n*=3).

was also good for perilla stalk extract, but perilla seed extract revealed a very low value.

Superoxide anion is a reduced form of molecular oxygen and has been implicated in the initiating oxidation reactions associated with ageing (Cotelle et al., 1996). Superoxide anions play an important role in formation of other ROS such as singlet oxygen, hydrogen peroxide and hydroxyl radical which induce oxidative damage in DNA, lipids and proteins (Pietta, 2000). We investigated the superoxide radical scavenging activity of perilla extracts using the nitroblue tetrazolium chloride-NADH-phenazine methosulphate (NBT-NADH-PMS) system. Decrease of optical density values against control is the indication of the presence of bioactive compounds possessing superoxide radical scavenging activity. The scavenging abilities on superoxide radical were good for perilla leaf and perilla seed extracts, but perilla stalk extract revealed a very low value (Figure 2).

Nakamura et al. (1998) previously reported that a high concentration of rosmarinic acid in *P. frutescens* leaf was found to have elevated superoxide radical scavenging activity. In addition, Gülçin et al. (2005) reported that the anthocyanins from *P. pankinensis* exhibited a high scavenging efficiency toward superoxide radicals. Therefore, phenolic compounds effectively exhibited antioxidative activity in the biological systems through the scavenging of O_2^-.

The reducing properties of natural components may have a reciprocal correlation with the antioxidant activities (Gülçin et al., 2003b). For the measurements of the reductive ability, the Fe^{3+}–Fe^{2+} transformation was investigated of perilla extract using the potassium ferricyanide reduction method. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of each antioxidant sample. Reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. Reducing powers of perilla extracts had high reducing activity in dose-dependent manner (Figure 3). The higher phenolic content in perilla leaf and perilla stalk extracts might account for the better results found in their reducing power and DPPH radical scavenging activity. Different studies have indicated that the antioxidant effect is related to the development of reductones (Shon et al., 2003). Furthermore, reductones such as ascorbic acid can react directly with peroxides and also with certain precursors and thereby, prevent peroxide formation. The reducing ability of various extracts might be due to its hydrogen-donating capacity, as described by Shimada et al. (1992). Therefore, the perilla extracts might contain reductones which could react with free radicals to stabilize and terminate free radical chain reactions.

Chelating agents were reported to be effective as secondary antioxidants because they can reduce the redox potential, thereby stabilizing the oxidized form of the metal ion (Gülçin et al., 2007). Measurement of the rate of color reduction allows estimation of the chelating activity of the coexisting chelator (Yamaguchi et al., 2000). Both perilla extracts and standard EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and capture ferrous ion before ferrozine. The perilla extracts showed significant iron binding capacity at all amounts (Figure 4). The results demonstrated moderate iron binding capacity, suggesting that the antioxidant activity may be related to the capacity for iron binding. The difference among all perilla extracts were significantly different ($p < 0.05$).

Chelating effect on ferrous ions of an antioxidant molecule prevents oxyradical generation and the consequent oxidative damage. Chelating ability was significant as they reduced the concentration of the catalyzing transition metal in lipid peroxidation (Duh et al., 1999). Since ferrous ions were the most effective pro-oxidants in food system (Yamaguchi et al., 1988), the moderate to high ferrous-ion chelating abilities of the various perilla extracts would be somewhat beneficial. These assays were used for establishing the abilities of perilla extracts to chelate and had important applications for the pharmaceutical and food industries.

Although the biological activity of perilla as well as its superior safety was well documented, but there has been little report on the quantitative presence of phenolic compounds in perilla (Peng et al., 2005; Meng et al., 2009). Polyphenolics display important role in stabilizing lipid oxidation associated with its antioxidant activity (Osakabe et al., 2002; Gülçin et al., 2003a). The main polyphenolic compounds has been proven to be rosmarinic acid, and there are small amounts of flavonoids and phenolic acids such as catechin, apigenin, luteolin, caffeic acid, and ferulic acid found in leaf and seed of *P. frutescens* (Ishikura, 1981; Aritomi et al., 1985; Masahiro et al., 1996). Polyphenolic compounds are a variety of antioxidant compounds that have been used as dietary supplements for the prevention of pathological diseases and for the improvement of human health conditions (Zhang et al., 2006). The bioactivity of phenolics may be related to their ability to scavenge free radicals, chelate metals and inhibit lipoxygenase (Decker, 1997).

Conclusion

The results demonstrated that the 50% aqueous–methanol extracts of perilla seed, leaf and stalk may have a significant effect on antioxidant and antiradical activities; especially perilla leaf has effective DPPH and superoxide radicals scavenging activities and reducing power. Hence, perilla leaf can be used as an easy accessible source of natural antioxidants, as a food supplement or pharmaceutical agent.

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Preliminary studies on decontamination of some dried herbal products by gamma irradiation

A. Adu-Gyamfi*, V. Appiah and J. Nketsia-Tabiri

Department of Food Science and Radiation Processing, Biotechnology & Nuclear Agric. Research Institute, Ghana Atomic Energy Commission, P. O. Box LG 80, Accra, Ghana.

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Seven dried herbal products (DHP) were decontaminated using gamma radiation. The microbial loads (total viable count, TVC) of the raw and irradiated products were determined using the methods of serial dilutions and pour plate. Based on international standards for microbial load, the effective decontamination doses were determined for the DHP. The range of TVC for the DHP was 10^5 to 10^9 cfu/g. Milled roots of *Cryptolepis sanguinolenta* and milled stems and leaves of *Desmodium adscendens* had the highest counts of 8.0×10^8 and 2.0×10^9 cfu/g, respectively. Powdered seeds of *Moringa olifera* and *Grifornia simplicifolia* and the seeds of *Voacanga africanus* had relatively low TVCs of 6.4×10^5, 6.6×10^6 and 1.3×10^6 cfu/g, respectively. Irradiation with medium doses of 2.5 to 7.5 kGy reduced microbial loads of the DHP by 3 to 6 log cycles. A dose of 10 kGy reduced the microbial load by 4 to7 log cycles and a dose of 15 kGy eliminated viable cells from all the DHP. Effective decontamination doses for the DHP were estimated to range from 2.5 to 10.0 kGy. Decontamination using gamma irradiation can improve the microbial quality and enhance the safety of DHP for both the domestic and export markets.

Key words: Dried herbal products, gamma irradiation, microbial decontamination, microbial load.

INTRODUCTION

Traditional medicines involving the use of herbal items, animal parts and minerals have been used by mankind for thousands of years (Kaptchuk, 2000; Kloss et al., 1939; Lewis 1984; Unschuld, 1985). These medicines are indispensable to many communities due to their accessibility and affordability (Orwa, 2002). According to the World Health Organisation (1998), about 70 to 80% of the world population, particularly in the developing countries, relies on non-conventional medicines mainly of herbal sources. In Ghana, traditional medicines are widely used by approximately 60 to 70% of the population in rural areas (Botwe, 1999). The Centre for Scientific Research into Plant Medicine has identified approximately 1,000 medicinal plants in Ghana, 40 of which are used in the treatments of 33 diseases such as asthma, malaria, jaundice, typhoid fever, diabetes, hypertension and anaemia (The Centre for Scientific Research into Plant Medicine (CSRPM), 2000).

In recent times, the use of herbal products has increased globally due to their effectiveness, low toxicity and minimal side effects (Fang and Wu, 1998). The economic benefit of herbal products is immense. Over-the-counter sales of herbal medicines is reported to be more than US$ 5 billion world-wide and the rural economy

*Corresponding author. E-mail: adugyamfi21@yahoo.com.

dealing with these products is also worth an estimated amount of US $ 600 million (Anon, 2001).

Despite the huge economic potential and health benefits, there is now growing concern about the purity and quality of herbal products in most countries. The products undergo long periods of crude storage and are frequently contaminated with microorganisms due to a lack of professional expertise on the part of traditional healers, collectors or producers. Quite high bacterial populations and the presence of potential toxigenic fungi have been reported for some local herbal teas (Owusu and Odamtten, 1999). Elsewhere, microbiological deterioration has been detected in herbal products in Nigeria (Okunlola et al., 2007) and dehydrated ginseng in Korea (Kwon, 1991; Lee, 1989; Fang and Wu, 1998).

In order to meet internationally accepted standards, herbal products should have acceptable microbial quality. Traditionally, sterilisation techniques using steam, methyl bromide, ethylene oxide and phosphine have been used to decontaminate herbal products (IAEA, 2008; European Pharmacopoeia, 2002; United States Pharmacopeia, 1995). Continual use of these techniques has been limited in many respects due to their inherent drawbacks. Steam sterilisation leads to loss of volatile and/or thermo-sensitive components, methyl bromide depletes the ozone layer and ethylene oxide as well as phosphine are now considered as carcinogens and environmental toxicants (Jacobs, 1995; Boess and Boegl, 1996; Ahmed, 1991). The use of gamma radiation in the decontamination of dehydrated materials such as herbs and spices is well documented (Eiss, 2001; IAEA, 1992; Farkas, 1988; ASTM, 1998). Studies in many countries have reported the potential of ionizing radiation in reducing microbial loads of a variety of herbal medicines to acceptable levels (Hilmy et al., 1981; Migdal and Owczarczyk, 1998; Fang and Wu, 1998). There is therefore the need to explore the use of gamma irradiation as a decontaminating method by the herbal products industry in Ghana. This could improve the hygienic quality and enhance the suitability of the products for both the local and international markets.

The objective of this study was to investigate the use of gamma irradiation in the decontamination of some local dried herbal products.

MATERIALS AND METHODS

Samples

Seven samples of dried herbal products used for the study were obtained from products submitted by some pharmaceutical companies and herbal products enterprises for contract irradiation at the Radiation Technology Centre (RTC) of Ghana Atomic Energy Commission. The samples were selected based on their widespread use in treating ailments such as malaria, fevers, cancers, hypertension, depression, asthma and rheumatism (CSRPM, 2000). Selection was also based on their potential for export and therefore the consequent need to improve their microbiological quality to meet international standards.

Milled roots of *Cryptolepis sanguinolenta*;
Milled leaves of *Lippia multiflora*;
Milled stems and leaves of *Desmodium adscendens*;
Powdered seeds of *Moringa olifera*;
Powdered seeds of *Grifornia simplicifolia*;
Seeds of *Grifornia simplicifolia*;
Seeds of *Voacanga africanus*.

Irradiation

Ten grammes of each product was packaged in a polyethylene pouch and sealed using a heat sealer (Heat Sealer, Desk Type: 300 m/m, Taiwan). The pouches were treated with medium irradiation doses of 2.5, 5.0, 7.5 and high irradiation doses of 10.0, 12.5 and 15.0 kGy at the RTC of Ghana Atomic Energy Commission using a Co^{60} source (SLL-515, Hungary) at a dose rate of 2.55 kGy/hr in air. The absorbed dose was confirmed by Fricke's dosimetry.

Determination of microbial load

After irradiation, both controls and irradiated products were analysed for their microbial load using the methods of serial dilution and pour plate. Five grammes of each sample was added to 45 ml peptone water (1% peptone + 0.5% NaCl) and placed on a mechanical shaker (Junior Orbital Shaker, Lab-line Instruments, USA) for 15 min. The mixture was then allowed to settle for about 5 min to allow coarse material to settle down. Microbial load determination was carried out on the supernatant by estimating the total viable counts (TVC) on Plate count agar (Oxoid, UK) at 36°C for 48 h using a colony counter (Stuart Scientific, UK) according to the methodology of APHA (1976). The TVC gives a quantitative idea about the presence of microorganisms such as bacteria, yeast and mold in the samples. For each product, the average of three estimations of duplicate plating was carried out, the mean microbial load and the range of the microbial load was determined.

RESULTS

As shown in Table 1, the range of microbial load (TVC) for the raw or unirradiated dried herbal products was 6.4×10^5 to 2.0×10^9 cfu/g. Milled roots of *C. sanguinolenta* and milled leaves and stems of *D. adscendens* had high counts of 8.0×10^8 and 2.0×10^9 cfu/g, respectively. Powdered seeds of *M. olifera* had a relatively low mean count of 6.4×10^5 with a range of 4.0×10^4 to 8.6×10^5.

Irradiation with medium doses from 2.5 to 7.5 kGy reduced mean microbial loads of the dried herbal products by 3 to 6 log cycles (Table 2). A dose of 10 kGy reduced mean microbial load of all the herbal products by 4 to 7 log cycles (Table 3). In the case of powdered seeds of *M. olifera* and *G. simplicifolia* as well as whole seeds of *G. simplicifolia* and *V. africanus*, an irradiation

Table 1. Mean and range of microbial loads of dried herbal product samples.

Dried Herbal Product	[a]Mean microbial load	[a]Range of microbial load
Milled roots of *Cryptolepis sanguinolenta*	[b]8.0×10^{8}	7.4×10^{8}–9.1×10^{8}
Milled leaves of *Lippia multiflora*	1.0×10^{6}	4.0×10^{4}–3.0×10^{6}
Milled stems and leaves of *Desmodium adscendens*	2.0×10^{9}	1.3×10^{8}–2.8×10^{9}
Powdered seeds of *Moringa olifera*	6.4×10^{5}	4.0×10^{4}–8.6×10^{5}
Powdered seeds of *Grifornia simplicifolia*	6.6×10^{6}	5.0×10^{4}–9.0×10^{5}
Seeds of *Grifornia simplicifolia*	1.1×10^{7}	4.0×10^{5}–1.2×10^{7}
Seeds of *Voacanga africanus*	1.3×10^{6}	1.0×10^{5}–1.4×10^{6}

[a]Total viable cells (cfu/g); [b]values are means of triplicate samples.

Table 2. Mean and range of microbial load of dried herbal products after gamma irradiation at medium doses.

Dried herbal product	[a]Microbial load		
	2.5 kGy	5.0 kGy	7.5 kGy
Milled roots of *Cryptolepis sanguinolenta*	[b]9.0×10^{6}[c] (8.5×10^{5}-9.5×10^{6})	1.8×10^{5} (5.0×10^{5}-1.5×10^{6})	1.0×10^{4} (7.0×10^{3}-1.2×10^{4})
Milled leaves of *Lippia multiflora*	2.0×10^{5} (9.0×10^{4}-3.0×10^{5})	5.0×10^{4} (9.0×10^{3}-9.5×10^{4})	4.0×10^{3} ($8.\times10^{2}$-6.0×10^{3})
Milled stems and leaves of *Desmodium adscendens*	6.0×10^{7} (5.0×10^{6}-9.5×10^{7})	5.0×10^{6} (2.5×10^{6}-8.0×10^{6})	4.0×10^{5}(9.0×10^{3}-2.0×10^{5})
Powdered seeds of *Moringa olifera*	1.3×10^{4} (1.0×10^{3}-2.0×10^{4})	3.5×10^{3} (2.5×10^{3}-6.0×10^{3})	3.6×10^{2} (<10-6.0×10^{2})
Powdered seeds of *Grifornia simplicifolia*	3.7×10^{3} (2.0×10^{3}-3.5×10^{3})	3.0×10^{2} (<10-4.0×10^{2})	[d]<10
Seeds of *Grifornia simplicifolia*	1.6×10^{5} (9.0×10^{3}-8.0×10^{5})	7.5×10^{3} (5.5×10^{3}-9.0×10^{3})	1.8×10^{2} (<10-3.0×10^{2})
Seeds of *Voacanga africanus*	2.2×10^{3} (1.6×10^{2}-3.0×10^{3})	1.0×10^{2} (<10-3.2×10^{2})	<10

[a]Total viable cells (cfu/g); [b] values are means of triplicate samples; [c] range of microbial load, [d]Viable cells not detected, limit of detection = 10 cfu/g.

dose of 10 kGy completely eliminated all viable cells from the samples. With the exception of milled roots of *C. sanguinolenta* and milled leaves of *D. adscendens*, a dose of 12.5 kGy completely eliminated all viable cells from the dried herbal products. A dose of 15 kGy eliminated viable cells from all the dried herbal products.

Generally, irradiation of all the samples of dried herbal products with medium and high doses resulted in approximately 3 to 7 log cycles reduction in the microbial loads (TVC) as compared to those of the control or unirradiated samples.

DISCUSSION

One of the biggest challenges of the herbal products industry is the lack of standardization in production protocols, resulting in products of varying qualities. This is largely due to the fact that the quality of herbal products is usually dependent on the professional expertise of the traditional healers, collectors or producers of the products. Effective drying, storage and packaging are critical stages in production protocols which determine the quality of the dried herbal products.

Table 3. Mean and range of microbial load of dried herbal products after irradiation at high doses.

Dried herbal product	[a]Microbial load		
	10.0 kGy	12.5 kGy	15.0 kGy
Milled roots of *Cryptolepis sanguinolenta*	[b]4.0×10^3 [c](2.6×10^2–5.4×10^3)	1.0×10^2 (<10–1.6×10^2)	[d]<10
Milled leaves of *Lippia multiflora*	4.0×10^2 (5.0×10^2–3.0×10^2)	<10	<10
Milled stems and leaves of *Desmodium adscendens*	2.0×10^3 (5.0×10^2– 3.5×10^3)	2.0×10^2 (<10–3.0×10^2)	<10
Powdered seeds of *Moringa olifera*	<10	<10	<10
Powdered seeds of *Grifornia simplicifolia*	<10	<10	<10
Seeds of *Grifornia simplicifolia*	<10	<10	<10
Seeds of *Voacanga africanus*	<10	<10	<10

[a]Total viable cells (cfu/g); [b] values are means of triplicate samples; [c] range of microbial load. [d]Viable cells not detected, limit of detection = 10 cfu/g.

The microbial loads of the dry herbal products recorded in this study were generally high and had a wide range (10^5 to 10^9 cfu/g). The observed differences in microbial loads recorded in this study could be due to factors such as differences in indigenous microflora, lack of standard processing procedures, contamination and defective packaging. Although these values compare well with total bacterial counts of 10^5 to 10^8 cfu/g reported in herbal teas (Wilkinson and Gould, 1996), they are clearly higher than the range of 10^4 to 10^5 cfu/g reported for some local herbal teas (Owusu and Odamtten, 1999) and the range of 10^2 to 10^4 cfu/g reported for herbal medicines in Nigeria (Okunlola et al., 2007).

International standards for herbal raw materials require that total aerobic bacteria and fungi should not exceed 10^5 and 10^3 cfu/g or ml, respectively (European Pharmacopoeia, 2007; WHO, 1998). However, the microbiological criteria for local herbal materials state that TVC should be less than 10^6 cfu/g (Ghana Standards, 1997). Since herbal raw materials are utilized in a manner similar to raw food, their microbial criteria should be of a higher standard to ensure a safety margin for these products. Significantly, all the dried herbal products assessed in this study did not meet the criteria of the international and local standards in terms of their microbial loads with the exception of the powdered seeds of *M. olifera*.

Fortunately, decontaminating the products using gamma radiation helped in meeting the required standards. Irradiation is one of the few processes that allow the attainment of high standards for foods and medical products through the destruction of bacteria and other microorganisms. The primary mechanism by which radiation kills microorganisms is by splitting water molecules into hydrogen (H^+), hydroxyl (OH^-) and oxygen (O^{-2}) radicals. These radicals react with and destroy or deactivate microbial components such as DNA, proteins and cell membranes (Niemira and Sommers, 2006; IAEA, 1982). Irradiation has the capacity to improve the hygienic quality and extend the shelf-life of various products thus enhancing their competitiveness on both domestic and export markets. From the results, the effective irradiation doses for reducing microbial loads of the samples to acceptable international standards were estimated as:

1. Powdered seeds of *G. simplicifolia and M. olifera* and seeds of *V. africanus* 2.5 kGy.
2. Milled leaves of *L. multiflora* and whole seeds of *G. Simplicifolia* 5.0 kGy.
3. Milled roots of *C. Sanguinolenta* 7.5 kGy.
4. Milled stems and leaves of *D. Adscendens* 10.0 kGy.

In comparison with the control or unirradiated samples, results of this study has shown that irradiation of the dried herbal products with medium to high doses considerably reduced their microbial loads to acceptable national and international standards. However, a wide range of effective irradiation doses (2.5 to 10.0 kGy) was required for decontamination of the dried herbal products to acceptable standards. This observation might be due to non-uniformity of the microbial loads and that underlines the need for good manufacturing practices (GMPs) in production protocols to ensure low microbial load of products and subsequently the use of low (effective) irradiation doses. It is noteworthy that 15 kGy eliminated all contaminating microflora in all the dried herbal products. A careful examination of the results further indicates that while a dose of 12.5 kGy reduced the microbial loads of all the samples to < 10^3 cfu/g, 10 kGy reduced the microbial loads to < 10^4 cfu/g. In a related study, irradiation doses between 7.5 to 10.0 kGy reduced the microbial load of a range of herbal tea and products to less than 10^3 cfu/g without any significant changes to the quantity and composition of volatile oils (Farkas, 1988). Studies by Migdal and Owczarczyk (1998) also

indicated a dose of 10 kGy effectively decreased microbial load of raw herbal products by 6 log cycles to 8.6 × 10 cfu/g. Similarly, a dose of 2 to 3 kGy reduced mycoflora population in some local herbal teas by 4 to 5 log cycles (Owusu and Odamtten, 1999).

Ghana, as a tropical country, has a rich biodiversity including 3,600 plant species of which over 2,900 are indigenous (MES, 2002). The country therefore has a high potential for production of herbal products considering the fact that an estimated 33 and 27% of drugs worldwide are derived, respectively from higher plants and lower plants/microbes (Essegbey, 2002). However, for the country to break into the global market for dry herbal products, it is important to improve and modernize the herbal medicine industry. Production of high quality herbal products through the use of irradiation could guarantee the country a huge export market just as in most Asian countries.

It is recommended that future studies should investigate the radiosensitivities of the different bacterial and fungal isolates of dried herbal products with the view to their elimination with low irradiation doses. Additionally, the impact of irradiation on the active phytochemicals of dry herbal products should be investigated.

Conclusion

The microbial load of dried herbal products is quite high probably due to deficiencies in production protocols. It is important to integrate GMP into production protocols of dried herbal products so as to generally improve their quality. Gamma irradiation effectively reduced the microbial loads of dried herbal products to acceptable national and international standards. The utilization of gamma irradiation by the herbal products industry could improve quality and guarantee access to lucrative global markets.

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Review

DNA markers in the authentication of Traditional Chinese Medicine

Ying Liu[1], Hannah Tan[2], Yuanling Qi[3], Xinmin Ruan [4]* and Naiyou Liu[4,5]

[1]College of Natural and Agricultural Sciences, University of California, Riverside, CA 92521, USA.
[2]Department of Surgery, Unversity of California, Los Angeles, CA 90095, USA.
[3]Department of Oncology, Weifang People's Hospital, Shandong 261041, China.
[4]Heart Center, Guangdong Provincial Hospital of Traditional Chinese Medicine, Guangdong University of Traditional Chinese Medicine, Guangzhou 510105, China.
[5]Department of Surgery, Cedars-Sinai Medical Center, David Geffen School of Medicine, University of California, Los Angeles, CA 90048, USA.

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Traditional Chinese medicine (TCM), with its multi-target effects, is gaining more and more attention all over the world due to its specific theory and long historical clinical practice. But the absence of an objective and accurate inspection system is frequently cited as one of the major hurdles for its modernization and globalization. Benefiting from modern molecular biology and polymerase chain reaction (PCR) techniques, DNA markers have become one of the most reliable methods for identification and authentication of Chinese medicinal materials. This paper reviewed the recent progress of DNA markers, including PCR-based markers, hybridization-based markers, sequenced-based markers, and DNA microarray in the authentication of TCM.

Key words: DNA marker, Traditional Chinese medicine (TCM), polymerase chain peaction (PCR).

INTRODUCTION

Traditional Chinese medicine (TCM) is an integral part of Chinese culture. It has been used in China for more than 20 centuries in the prevention and healing of human diseases. Due to its long history of clinical use, reliable therapeutic efficacy, unique treatments, and systemic theories, TCM is getting increasing global attention; furthermore, an increasing variety of natural bioactive compounds have been developed from TCM by many big, worldwide pharmaceutical companies to meet the contemporary trend of "back to nature" (Shaw et al., 2002). However, ensuring the therapeutic effects of TCM is a challenging task because of the nature of multi-component mixtures. Substitutes and adulterants of TCM materials may interfere with their therapeutic effects, even resulting in poisoning. Thus, one major hurdle that might impair TCM's potential future as "medicine of choice" is the lack of standardization.

Traditionally, identification of TCM involves its morphology, histological characteristics, and/or content determination, which needs a lot of time and labor. In recent years, many significant analytical advancements and tools were made in relation to this particular problem. One of the most reliable methods of identification of TCM is DNA analysis. DNA markers for TCM identification are less affected by age, physiological conditions, environmental factors, as well as harvest, storage and processing methods (Jiang et al., 2010). Further, only a small amount of DNA is needed, and the physical form of the sample does not restrict detection. This is extremely useful when the TCMs are expensive or short in supply.

*Corresponding author. E-mail: xinmin.ruan@gmail.com. Tel: 01-310-926-6734. Fax 01-310-423-7662.

In this article, we review versatile DNA-based molecular markers that can be employed to analyze DNA for quality assurance, control, and authentication of TCM.

PCR based markers

PCR technique permits the amplification of any sequence of up to 40 kb of DNA, even in samples containing only minute quantities of DNA. Thus, the PCR technique is extremely useful in studying the genetic similarity or dissimilarity of TCM. Following amplification, the polymerase chain reaction (PCR) products are fractionated on agarose, polyacrylamide, or another type of gel matrix before it is detected by ethidium bromide (EtBr), autoradiography, or fluorescence by using a fluorescence labeled primer. To date, a variety of PCR-based methods have been established for the authentication of TCM.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

RFLP was the first DNA profiling technique inexpensive enough to see widespread application. However, it is slow and needs a lot of labor. Also, it requires a large amount of sample DNA (Saiki et al., 1985). Therefore, PCR-RFLP analysis, which consumes a minute amount of DNA, is much more suitable for TCM (Meyer et al., 1995). By using PCR-RFLP, a defined DNA fragment is first amplified by PCR, then it is digested by a certain restriction endonuclease to generate a restriction polymorphic profile unique to the species concerned (Shaw et al., 2002). Theoretically, the region for PCR amplification should be highly conserved among species so that it can be easily amplified. The analysis of sequence variation in the regions such as ribosomal DNA (rDNA) and a large subunit of ribulose-1,5-bisphosphate carboxylase (rbcl) has become an effective method for identification of medicinal herbs. The PCR-RFLP of rDNA or rbc1 has been successfully carried out on *Glehnia* (Mizukami et al., 1993), *Epimedium* (Nakai et al., 1996), *Atractylodes* (Mizukami et al., 1996; Cheng et al., 1997), *Panax* (Ngan et al., 1999), *Codonopsis* (Fu et al., 1999), *Dendrobium* (Zhang et al., 2005), *Fritillaria* (Wang et al., 2005; 2007), *Sinopodophyllum* and *Dysosma* (Gong et al., 2006), *Ganoderma* (Zhou et al., 2008), *Akebia* (Kitaoka et al., 2009) and *Lonicera* (Peng et al., 2010).

Random amplification of polymorphic DNA (RAPD)

RAPD is a PCR based analysis, but it only amplifies segments of DNA which are essentially unknown. This technique uses a single arbitrarily chosen primer of 10 nucleotides long as both the forward and reverse primers in a PCR reaction (Williams et al., 1990). Typically the scientist performing RAPD creates several arbitrary primers, and then proceeds with the PCR using a large template of genomic DNA to amplify fragments. By resolving the resulting patterns, a semi-unique profile can be obtained from a RAPD reaction. Other similar approaches include Arbitrarily-Primed PCR (AP-PCR) (Welsh and McClelland, 1990), and DNA amplified fragments (DAF) (Caetano-Anolles et al., 1991a, b). The only difference among these approaches is that AP-PCR uses primers approximately 20 nucleotides long, while DAF uses a primer which is 5-8 nucleotides long. In the past 20 years, RAPD and related methods have been extensively applied to study the genetic similarity or dissimilarity of TCM incuding *Glycyrrhiza* (Yamazaki et al., 1994), *Panax* (Shaw and But, 1995), *Cannabis* (Gillan et al., 1995; Jagadish et al., 1996; Shirota et al., 1998), *Clematis* (Zhang et al., 1996), *Elephantopi* (Cao et al., 1996a), *Tataxaci* (Cao et al., 1996b), *Snakes* (Wang and Zhou, 1996; 1997), *Coptis* (Cheng et al., 1997), *Indigofera* (Zhang et al., 1997), *Asari* (Huang et al., 1998), *Liriope* (Wu et al., 1998), *Anoectochilus* (Cheng et al., 1998), *Perilla* (Ito et al., 1998), *Codonopsis* (Fu et al., 1999), *Trichosanthes* (Wang et al., 1999), *Scutellaria* (Hosokawa et al., 2000), *Dysosma* (Fu et al., 2000), *Atractylodes* (Chen et al., 2001; Guo et al., 2001), *Magnolia* (Guo et al., 2001), *Paeonia* (Zhou et al., 2002), *Rehmannia* (Cheng et al., 2002), *Cortex Magnoliae Officinalis* (Liu et al., 2004), *Morinda* (Ding et al., 2006), *Pogostemon* (Pan et al., 2006), *Selaginella* (Li et al., 2007), and *Lycium* (Zhang et al., 2001; Sze et al., 2008).

Amplified fragment length polymorphism (AFLP)

The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA (Vos et al., 1995). Developed in the early 1990s, AFLP consists of three steps: (1) Digestion of total genomic DNA by restriction enzymes, followed by ligation of adaptors to the sticky ends of the restriction fragments. (2) Selective amplification of a subset of the restriction fragments by using primers complementary to the adaptor sequence and restriction site sequence. (3) Visualization of the amplified fragments on denaturing polyacrylamide gels, either through either autoradiography or fluorescence methodologies (Zabeau and Vos, 1993). The AFLP technology is highly sensitive and reproducible, capable of detecting various polymorphisms in different genomic regions simultaneously. Thus, AFLP has become successfully used for the identification of genetic variation in strains or closely related species of TCM, such as *Panax quinquefolius* and *Panax ginseng* (Shaw et al., 1998), *Fritillaria* (Cai et al., 1999), *Panax* (Hong et al., 2005), *Radix* (Chen et al., 2005), and *Magnolia* (He et al., 2009) etc.

Simple sequence repeats (SSRs) and inter-simple sequence repeat (ISSR)

SSRs, also referred to as microsatellites, or sometimes short tandem repeats (STRs), are short sequences of nucleotides (2 to 6 units in length) that are repeated in tandem (Grist et al., 1993). SSRs can be amplified for identification by PCR, using the unique sequences of flanking SSRs regions as primers. Developed by Zietkeiwitcz et al. (1994), ISSR is a genomic region between SSRs. The complementary sequences of two neighboring SSRs are used as PCR primers, thus the variable region between them is amplified (Zietkeiwitcz et al., 1994). Both SSBs and ISSR have proven to be versatile molecular markers for assessing genetic relatedness in TCM populations of the same species such as *Primula* (Nan et al., 2003), *Vitex rotundifolia* (Hu et al., 2008), *Cistanche* (Shi et al., 2009), and *Vitex trifolia var. simplicifolia* (Liu et al., 2010b).

Direct amplification of length polymorphisms (DALP)

DALP technique uses an arbitrarily primed PCR to produce genomic fingerprints and to enable direct sequencing of DNA polymorphisms in virtually any species (Desmarais et al., 1998). Compared to arbitrarily primed fingerprinting, it detects a larger number of polymorhic loci and simplifies the procedures for recovery of polymorphic DNA bands. Dr. Shaw's group has successfully adopted DALP technique to authenticate *P. ginseng* and *P. quinquefolius* from different farms (Ha et al., 2002).

Other PCR-based methods

Many other alternative PCR-based techiniques have been developed. Single-strand conformation polymorphism (SSCP), or single-strand chain polymorphism detects the changed migration rate of DNA molecules of identical length due to sequence-dependent, differential intramolecular folding of ssDNA by non-denaturing gel electrophoresis (Orita et al., 1989). PCR-SSCP technique has been used to differentiate *Cannabis sativa* and *Humulus lupulus* (Kohjyouma et al., 2000), and to identify *Mycobacterium* (Jiang et al., 2009). Denaturing gradient gel electrophoresis (DGGE) is a molecularfingerprinting method that separates polymerase chain reaction (PCR)-generated DNA products by applying them to an electrophoresis gel that contains a denaturing agent (Fischer and Lerman, 1983). DGGE-RAPD analysis, combining DGGE and RAPD, was proved to be highly reproducible and gives a higher level of polymorphism and consequently more markers compared to RAPD only (Dweikat et al., 1993; Bahieldin et al., 2006). This will be a useful tool for TCM authentication.

HYBRIDIZATION-BASED MARKERS

By using hybridization-based markers, DNA profiles are visualized by hybridizing the restriction enzyme-digested DNA, to a labeled probe, which is a DNA fragment of known origin or sequence. Restriction fragment length polymorphism (RFLP) is a typical molecular marker based on the differential hybridization of probe DNA to DNA fragments cleaved with restriction enzymes. RFLP is most widely used in genome mapping, marker-aided breeding, systematic, and evolution studies (Kochert, 1994; Shaw et al., 2002). SSRs or microsatellites, which are another representative of hybridization-based marker technology are generally less than 6 bp in length and are repeated from a few to thousands of times. The regions flanking the microsatellites can be amplified, providing co-dominant sequence-tagged sites (STS) or a repetitive sequence to act as a probe. The methodology has been derived from RFLP, and specific fragments are visualized by hybridization with a labeled microsatellite probe (Litt and Luty, 1989). SSRs have been used to generate DNA fingerprints in TCM populations of the same species such as *P. ginseng* and *P. quinquefolius* (Leung and Ho, 1998), *Primula* (Nan et al., 2003), *Vitex rotundifolia* (Hu et al., 2008), *Cistanche* (Shi et al., 2009), and *V. trifolia var. simplicifolia* (Liu et al., 2010b).

SEQUENCING-BASED MARKERS

DNA sequencing is a definitive means for the identification of TCM. The reducing cost of DNA sequencing has led to the availability of large sequence data sets derived from the discovery of whole genome sequencing and large scale expressed sequence tag (EST), which is a valuable source of new genetic markers. A representative region for sequencing is the ITS from ribosomal RNA genes (known as ribosomal DNA or rDNA). Eukaryotic rDNAs are found as parts of repeat units that are arranged in tandem arrays, located at the chromosomal sites known as nucleolar organizing regions (NORs). Each repeat unit consists of a transcribed region (having genes for 18S, 5.8S and 26S rRNAs and ITS. The length and sequences of ITS region of rDNA repeats are believed to be fast evolving and therefore may vary, making this region a useful sequence for phylogenetic studies (Baldwin et al., 1995; Mitchell and Wagstaff, 1997). Generally, sequence homologies within species are high while those between species or families are low, indicating the ITS region can be used as a marker from interspecific level to family level (Shaw et al., 2002). Researchers have sequenced ITS region for the authentication of TCM, such as *Codonopsis* (Fu et al., 1999), *Panax* (Ngan et al., 1999), *Dendrobium* (Lau et al., 2001; Ding et al., 2002; Xu et al., 2006), *Alpinia* (Zhao et al., 2001), *Hericium* (Lu et al., 2002), *Gentiana* (Ji et al., 2003; Liu et al., 2010a), *Artemisia* (Kim et al., 2004),

Ephedra (Long et al., 2004; Kakiuchi et al., 2006), *Fritillaria* (Wang et al., 2005), Euphorbia (Xue et al., 2007a), *Croton* (Xue et al., 2007b), *Pogostemon* (Zhang et al., 2007), *Nelumbo* (Lin et al., 2007), *Psammosilene* (Liu et al., 2008), *Fallopia* (Zheng et al., 2008), *Cimicifuga* (Xue et al., 2009), and *Lonicera* (Peng et al., 2010).

Researchers also have sequenced regions of DNA (18S, 5.8S, 26S and 5s rDNAs etc.) for verifying TCM, including *Cordyceps* (Ito and Hirano, 1997), *Fritillaria* (Cai et al., 1999; Cai et al., 2001; Li et al., 2003), *Astragalus* (Ma et al., 2000), *Crocus* (Ma et al., 2001), *Notoginseng* (Cao et al., 2001), *Alpinia* (Zhao et al., 2002), *Adenophorae* (Zhao et al., 2003a), *Angelica* (Zhao et al., 2003b), *Panax* (Zhang et al., 2006), and *Curcuma* (Cao et al., 2010). Now, with a further decrease in the cost of sequencing, we expect that DNA sequencing will become a more common means for the authentication of TCM.

DNA MICROARRAY

DNA microarray was developed in response to the need for a high-throughput, efficient, and comprehensive strategy that can simultaneously measure the expression of all the genes, or a large defined subset, encoded by a genome. It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, known as probes, which are used to hybridize DNA targets labeled by fluorophore-, silver-, or chemiluminescence (Schena et al., 1995, 1996). DNA microarray based technology can be useful as an efficient, accuratem tool for the identification and authentication of TCM (Chavan et al., 2006). Although DNA microarray technology has huge potential for authentication and quality control of TCM, it is still in the exploratory stage and needs validation by other biological experiments. Standardized, sensitive, reproducible microarray platforms, databases, and visualization methods for expression profiles are needed.

CONCLUSION

DNA technology provides an independent and reliable approach for the differentiation of Chinese medicinal individual, species and population, homogeneity analysis, and detection of adulterants. At present, the authentication of TCM is moving towards a more comprehensive and overall direction. More and more system biology methods, including metabonomics, genomics and proteomics, are introduced to this area. One of the immediate tasks for researchers in this field is to build a reference library of TCM with genetic information (such as DNA sequences and fingerprints), in order to promote the further development of more scientific, effective, and practical DNA methods for the authentication of TCM.

ABBREVIATIONS

AFLP, Amplified fragment length polymorphism; **AP-PCR,** arbitrarily-primed PCR; **DAF,** DNA amplified fragments; **DALP,** direct amplification of length polymorphisms; **DGGE,** denaturing gradient gel electrophoresis; **ISSR,** inter-simple sequence repeat; **ITS,** internal transcribed spacers; **PCR,** polymerase chain reaction; **PCR-RFLP,** polymerase chain reaction-restriction fragment length polymorphism; **RAPD,** random amplification of polymorphic DNA; **RFLP,** restriction fragment length polymorphism; **SSCP,** single-strand conformation polymorphism; **SSRs,** simple sequence repeats; **TCM,** traditional Chinese medicine.

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Full Length Research Paper

Wild edible fruits used by Badagas of Nilgiri District, Western Ghats, Tamilnadu, India

R. Sathyavathi* and K. Janardhanan

Department of Botany, Bharathiar University, Coimbatore, 641 046, India.

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Nilgiri means "Blue Mountains". The entire area of the Blue Mountains constitutes the present district of Nilgiri. It was originally a tribal land and was occupied by the Todas, Kotas, Kurumbas, Kattunayakkas and Panyas. Among them, the Badagas are one of the major communities in the district who reside in the mountain as long as other tribal population. The weather in this district favours the growth of wild edible fruit crops. There are many edible fruits existing naturally in forest as well as in cultivable areas. These fruit plants are playing vital role in providing nutritional and economic values in the rural areas. This paper lists 30 wild edible fruits used by the Badagas of Nilgiri District for their conservation and cultivation purpose. But the commercial importance and the market value of these wild fruits is unknown to them. Hence this study is being taken to conserve those wild edible fruits and cultivate in large scale, to uplift their economic status in near future.

Key words: Badagas, wild edible fruits, conservation, Nilgiris.

INTRODUCTION

India is one of the twelve mega diversity in the world. The Nilgiris falls within the Western Ghats and is considered to be a home of more then 3500 species of wild plants. The forest of the Nilgiris provide a large number of plants whose fruits, seeds, tubers, shoots etc. make an important contribution to the diet of the people, particularly those living near forest and other rural areas. These plants not only provide inexpensive food but several other useful products like medicine, fiber, fodder, dyes, etc. Historically, fruits and vegetables have been used as medicinal agents. Wild fruits contain a significant level of biological active components that fruits are rich source of vitamins, minerals and other nutrients, but in contrast, wild fruits are often viewed with distrust. With the use of wild fruits not known, these people destroy the wild crop field for cultivation purpose.

Recent phytochemical investigations in fruits have attracted a great deal of attention, with mainly concentrations on their role in preventing diseases caused as a result of malnutrition. Though there are many wild fruits in this region, there is no proper collection, improvement and agro-techniques for these crops. Hence much emphasis should be given to exploration and collection, *in situ* or *ex situ* conservation, studying nutritional and anti-nutritional properties, product development and marketing (Sankaran et al., 2006).

The fruits are nature's gift to mankind. These wild fruits are chief source of vitamins, minerals and proteins. These constituents are essential for normal physiological well being and help in maintaining healthy state through development of resistant against pathogens (Bal, 1997). Rathod and Valvi (2011) studied the antinutritional factors of some wild edible fruits from Kolhapur district. Cyril et al. (1993) reported less known edible fruit-yielding plants of Nilgiris. It is known that the intake of wild crop reduces the rate of diseases and increases the rate of resistance and ageing. Fruit plants are playing a vital role in providing nutritional and economical security to the poor

*Corresponding author. E-mail: dr.r.sathyavathi@gmail.com.

Figure 1. Map of study area.

mass in rural areas but the commercial importance and market value of these wild fruits in unknown to them. Hence the present study was made to list out identification of underutilized wild edible fruits used by the indigenous community of Badagas from Nilgiri hills, and to conserve those plants for their future generations.

Study area

The Nilgiris of Blue Mountains are some of most picturesque mountain ranges situated mainly in the Northwestern corner of Tamilnadu in South India. They are surrounded in the North by the state of Karnataka and West and Southwest by Kerala and lie between 11° 12' to 11° 43' and 76° 14' to 77° 11' E, and are meeting ground (or) nexus of three mountains system of Peninsular India, Western Ghats, Southern Ghats and Eastern Ghats. The Nilgiris comprise the second highest peak in India situated in the South of Himalayas. Udagamandalam (Ootacamund), the most popular hill station in southern India, is situated in a broad undulating valley at the foot of Doddabetta (Figure 1).

Anthropolgy of Badagas

The name Badagas (corrupted to 'burgher' by the early European visitors to the hills) is similar to the word vadaga that means 'Northern', and the Badaga of the plateau are the descendents of canaries who immigrated to Karnataka. Unlike the tribes like Todas, Kotas and Kurumbas whose number never exceeded about a thousand, the Badagas are the largest social group in the Nilgiris, the present population of badagas is estimated at 1,50,000, spreading over 370 hamlets (www.badaga.org). The Badagas basically were included under the list of scheduled tribes till the 1971 census. After that, they were included under backward community list, making them eligible to avail 27% reservation in both central and state government jobs (www.badaga.org).

The badaga are split into a six-sub division: Wodeya, Haruva, Athikari, Kanakka, Badaga and Toreya. The first two sub divisions and several in the third sub division are purely vegetarian and are Lingayats and others are Saivites.

MATERIALS AND METHODS

The present work is the outcome of intensive field studies undertaken in hamlet inhabited by Badaga community. Explorative field trips were regularly made once in a month of the year to all habitations to elicit information on medicinal plants used to treat various ailments. All the medicinal important plants were collected as per the information given by the informants. The plant specimens collected were identified with the help of Flora of Presidency of Madras (Gamble and Fischer, 1915), Flora of Nilgiri and Pulney Hills (Fyson, 1915) and Flora of South Indian Hill Station (Fyson, 1977), and confirmed with the authentic herbarium of Government of India, Botanical survey of India, Southern circle, Coimbatore and survey of medicinal plant collection unit, Government Arts College campus, Ootacamund. Field numbers were given for all the collected plant specimens. Herbarium specimens were prepared according to the method of Jain and Rao (1977). All the prepared herbarium specimens were deposited in the Department of Botany, Bharathiar University, Coimbatore.

Table 1. List of wild edible fruits used by Badagas of Nilgiris.

S/No	Plant name	Family	Local name	Therapeutic uses	Habit
1	*Annona reticulata*	Annonaceae	Seetha annu	Diarrhoea, Edible	Tree
2	*Berberis tinctoria*	Berberidaceae	Jakkal annu	Jaundice, Stomachache, tooth ache	Erect shrub
3	*Carissa paucinervia*	Apocynaceae	Kavill annu	Quenching thirst	Spinous shrub
4	*Cyphomandra betacea*	Solanaceae	Thambitai	Rich in Vitamins	Tree
5	*Elaeagnus kologa*	Elaeagnaceae	Kolanganannu	Heart pain, fever	Straggling shrub
6	*Elaeocarpus tectorius*	Elaeocarpaceae	Bikkannu	Skin allergy	Tree
7	*Ficus carica*	Moraceae	Seeme atti	Chest pain, epilepsy, asthma	Tree
8	*Ficus racemosa*	Moraceae	Athi annu	Anti-diuretic	Small tree
9	*Ficus exasperata*	Moraceae	Maramthinai athi	Fertility	Small tree
10	*Fragaria nilgherrensis*	Rosaceae	kadannu	Edible	-
11	*Morus alba*	Moraceae	Kambilipoochi	Purify blood, treat constipation and diabetes	Climber
12	*Oxalis latifolia*	Oxalidaceae	Ullamajige	Paralysis	Bulbous herb
13	*Passiflora edulis*	Passifloraceae	Odey annu	Head ache	Climbing shrub
14	*Physalis minima*	Solanaceae	Kotharannu	Intestinal pain	Herb
15	*Physalis peruviana*	Solanaceae	Pitlannu	Vomiting	Herb
16	*Polygonum chinensis*	Polygonaceae	Kappu annu	Paralysis, thirst	Sub herb
17	*Psidium guajava*	Myrtaceae	Koyya annu	Diarrhea	Tree
18	*Rodomyrtis tomentosa*	Myrtaceae	Thavutte annu	Dental diseases	Shrub
19	*Rubus ellipiticus*	Rosaceae	Thuppa mulli	Easy digestion, paralysis	Shrub
20	*Rubus molucanus*	Rosaceae	Mulli annu	Easy digestion, paralysis	Shrub
21	*Rubus racemosus*	Rosaceae	yemmemulli	Easy digestion, paralysis	Shrub
22	*Solanum nigrum*	Solanaceae	Gakaiannu	Diabetes, tuberculosis, diarrhea and skin diseases	Herb
23	*Solanum sismbrifolium*	Solanaceae	Thomba annu	Antifertility, vermifuge	Shrub
24	*Syzygium arnottianum*	Myrtaceae	Nerli annu	Toothache	Tree
25	*Syzygium calophyllifolium*	Myrtaceae	Kadu nerli	Toothache	Tree
26	*Syzygium cumini*	Myrtaceae	Nerli annu	Toothache	Tree
27	*Taxillus buceatus*	Loranthaceae	Gannaiannu	Skin allergy	Parasitic herb
28	*Todalia asiatica*	Rutaceae	Massikai	Skin allergy	Shrub
29	*Zizyphus rugusa*	Rhamnaceae	Anthonannu	Monorrhigia, diarrhoea	Shrub
30	*Celtis cinnamomea*	Ulmaceae	Adhuve annu	Cuts and wounds	Tree

*Annu denotes fruit.

RESULTS AND DISCUSSION

In this present study, 30 wild edible fruits eaten by Badagas have been recorded and tabulated according to their plant name(s), family, local name, therapeutic uses and habits (Table 1), in which Solanaceae, Myrtaceae and Rosaceae each of 5 species and Moraceae 4 and the rest are represented with one or two species. The study indicates the extensive use of wild edible fruits by Badagas in Nilgiri hills of Western Ghats, Tamilnadu. Some of the wild edible plants are used for medicinal as well as some religious purpose. *Eleagnus kologa* leaves are kept in front of the house to protect it from evil spirits and *Eleacocarpus tectorius* is served as sacred trees of Badagas, Todas, Kotas and Krumbas. *Rhodomyrtis tomentosa* stem and *Rubus ellipticus* leaves are used in important festivals like Oppu when they pay homage to cows which serve all their needs while *Rubus molucanus* leaves are for when they offer the agricultural produce to God. *Syzygium cuminii* tree is used in all religious purpose for all tribes of Nilgiris.

Traditional and indigenous medical knowledge of plants, both oral and codified, are undoubtedly eroding (Mujtaba and Khan, 2007). There is a huge genetic diversity which can be used for creating the gene bank (field and *in vivo*), and the collection, conservation and utilization of these crops and standardization of agro-techniques is essential for their profitable cultivation and

Figure 2. (a) Vegetational slopes in Nilgiris, (b) author with Badagas, (c) author collecting information about both medicinal plants and Badagas anthropology, (d) *Carissa paucinervia* A.DC., (e) *Eleagnus kologa* Schult., (f) *Psidium duajava* Linn., (g) *Solanum sismbrifolium* Linn.

maximum yield realization in these crops (Sankaran et al., 2006) (Figure 2).

Conclusion

The Nilgiri hill is fast growing for eco-tourism and other economical purpose, and this threatened their knowledge. This is indicative of the vast repository of knowledge of plants that is still available for global use, provided of course that it does not get lost before it can be tapped or documented. In the present scenario, traditional knowledge system in our country is fast eroding and there is an urgent need to inventories, record all ethno-botanical and cultural information among the diverse ethnic communities before the traditional cultures are completely lost. Therefore, documentation of information will help in conserving the knowledge. A comprehensive database of the plants used for various purposes could be saved for the forthcoming generations. In such cases, laboratory investigations and clinical trials are suggested to validate the therapeutic properties of these wild edible fruits for effective and safe use.

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Full Length Research Paper

DNA isolation and optimization of SRAP-PCR condition for endangered *Polyporus umbellatus*

Yuejin Zhang, Yalin Qin, Zhen Wang, Lijun Guo, Xin Chen, Zongsuo Liang and Hongbo Guo*

Shaanxi Research Center of TCM Fingerprint and NP Library, College of Life Sciences, Northwest A&F University, yangling 712100, PR China.

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To provide a fast genetic diversity survey of endangered *Polyporus umbellatus* (Pers.) Fries for immediate conservation, DNA isolation and optimization of polymerase chain reaction (PCR) assay of sequence-related amplified polymorphism (SRAP) were investigated. Due to high amount of polysaccharide contained in mycelium, three special approaches were adopted to eliminate it during DNA isolation, including adding RNAiso-mate for plant tissue buffer to mycelium powder, ethanol to DNA extraction buffer and 5% NaCl solution to the mixture of isoamyl alcohol and DNA deposit solution. Based on screening design, the optimal SRAP-PCR condition was a total volume of 25 µl containing 20 ng of DNA template, forward primer 0.4 µM, reverse primer 0.4 µM, 1× Taq MasterMix and the best annealing temperature was 35/50°C for each primer combination. According to our optimal SRAP-PCR condition, forty-nine out of eighty-one primer combinations were chosen for their high clarity and repetition in all samples.

Key words: DNA isolation, sequence-related amplified polymorphism (SRAP), optimal polymerase chain reaction (PCR) condition, Chuling, *Polyporus umbellatus*.

INTRODUCTION

Chuling (*Polyporus umbellatus* (Pers.) Fries), a well-known medicinal fungus, is widely used in China and its sclerotium has been officially listed in China Pharmacopoeia as diuretic (National Committee of China Pharmacopoeia, 2010). However, with the quick growth of commercial demands for this medicinal material in recent years, excessive exploitation has severely shrunk its natural resources and it has been listed as endangered species in China Red Book (Li, 2008). To date, previous studies mainly focus on the resources distribution, components extraction, pharmacological properties and the relationship with its companion fungus (Xu, 1997; Xing and Guo, 2004; Yuan et al., 2004; Kikuchi and Yamaji, 2010; Zhao et al., 2010), and no effort has been made to its genetic diversity for immediate conservation of wild resources and future cultivar breeding.

Fortunately, DNA markers make a fast survey of a species' genetic diversity and the sequence-related amplified polymorphism (SRAP), a new molecular marker first introduced by Li and Quiros (2001), has several advantages such as simple, reproducible and easy isolation of fragments for sequencing. Now it has widely applied in genetic diversity analysis, genetic linkage map construction and molecular identification (Ren et al., 2010; Wang et al., 2012).

To conserve wild genetic resources of this medicinal fungus as soon as possible, a previous mycelial growth and polysaccharide content of seven wild *P. umbellatus* strains collected from seven provinces of China have been evaluated by our lab (Zhang et al., 2010). Here, the present study aimed to: (1) form an improved DNA isolation method for *P. umbellatus*; (2) obtain an efficient protocol of SRAP-polymerase chain reaction (PCR)

*Corresponding author. E-mail: zouguoge@yahoo.com.cn. Tel/Fax: 86-29-8709-2262.

Table 1. The isolated DNA purity and yield from 100 mg fresh mycelium of *Polyporus umbellatus* by different isolation methods.

DNA isolation method	A260/A280	A260/A230	Yield (mg g^{-1})
CTAB method reported by Han 2008[a]	1.44 ± 0.09	0.34 ± 0.03	13.03 ± 0.02
Present method with RNase treating 1 h[a]	1.92 ± 0.03	1.98 ± 0.01	149.65 ± 0.03
Present method with RNase treating 30 min[a]	2.08 ± 0.02	2.08 ± 0.01	45.45 ± 0.02

[a]Mean of eight samples from three independent experiments with standard deviation.

condition; and (3) screen primer combinations by using the optimal SRAP-PCR system.

MATERIALS AND METHODS

Sclerotium

Eight wild sclerotium strains of *P. umbellatus* were collected from seven provinces of China, including Shaanxi, Henan, Heilongjiang, Sichuan, Yunnan, Hebei and Hubei. They were identified and preserved at local laboratory in each region after collection.

DNA isolation

The activation culture of eight original sclerotium strains and their liquid culture were followed by our previous work (Zhang et al., 2010). The activated mycelium on Potato Dextrose Agar (PDA) medium was transplanted to liquid medium and cultivated at 25°C for 7 days under dark condition. The mycelium was washed with ddH_2O followed by filtering with sterile gauze and then was stored at -20°C for use after water absorption with sterile filter parer. Total genomic DNA was extracted from 100 mg fresh mycelium by using the modified cetyltrimethyl ammonium bromide (CTAB) method (Han, 2008).

In a pre-chilled mortar and pestle, 100 mg fresh mycelium was finely ground using liquid nitrogen, and quickly transferred to 2 ml RNAiso-mate for plant tissue buffer to primarily eliminate polysaccharide. The mixture was ground again untill the solution became transparent after the powder completely dissolved in the kit buffer, followed by centrifugation of the mixture at 12,000 ×g for 5 min at 4°C. A 700 µl extraction buffer pre-warmed at 65°C for 5 min was added to the upper aqueous phase and the mixture was shaken slightly for 4 times when incubating at 65°C for 1 h. The 700 µl phenol:chloroform:isoamyl alcohol (25:24:1) was added to it and mixed well by invert mixing. The contents were centrifuged at 13,000 ×g for 10 min at 4°C. Two microlitres RNase was added to the upper aqueous phase and the mixture was maintained at 37°C for 1 h. Another 700 µl phenol:chloroform:isoamyl alcohol was added to the mixture and mixed well, followed by centrifugation at 13,000 ×g for 10 min at 4°C. Equal volume of chloroform:isoamyl alcohol (24:1) was added to the upper aqueous phase and mixed well, followed by 13,000 ×g for 10 min at 4°C. The two volumes of isoamyl alcohol were added to the collected upper phase. The pellet was collected by centrifugation (12,000 ×g for 2 min at 4°C) after incubating the tubes at -20°C for 30 min and then washed twice with 75% ethanol, and centrifuged at 12,000 ×g for 1 min at 4°C. The pellet was re-suspended in 30 µl ddH_2O water after evaporation of ethanol.

DNA quantifications were performed by UV-spectrophotometer at 260 and 280 nm, and the purity was then determined by calculating the ratio of absorbance at 260 nm to that of 280 nm (OD_{260}/OD_{280}). DNA concentration and purity was also determined by electrophoresis on 0.7% agarose compared with dry matter (DM) 15000 DNA marker. The DNA solution was then diluted in 30 µl sterile distilled water to different concentrations for screening use.

Optimization of SRAP-PCR condition

Based on the report of Li and Quirous (2001), SRAP-PCR amplification was performed with 1× Taq MasterMix (Kangwei, Beijing) in a total volume of 25 µl. Another three factors including DNA template, primer combination concentration and annealing temperature were evaluated. DNA template included five concentrations: 10, 15, 20, 25 and 30 ng. Primer combination concentration included five grads: 0.24, 0.32, 0.40, 0.48 and 0.56 µM. Annealing temperature contained four combinations: 35/48, 35/50, 32/50 and 30/50°C. The PCR was performed in a PTC200 thermocycler (MJ Research Inc, Watertown, Mass). A denaturation period of 5 min at 94°C was followed by 5 cycles of 1 min at 94°C, 60 s at different annealing temperature and 1 min at 72°C, and then 35 cycles of 1 min at 94°C, 60 s at different annealing temperature and 1 min at 72°C with 10 min at 72°C for final extension. A 6 µl aliquot of the amplification products was separated by electrophoresis in 2.0% agarose gel in 1× TAE buffer and the DNA fingerprints were photographed by an automatically imaging system.

RESULTS

DNA isolation and detection

White deposit pellet was obtained in all strains by using the improved CTAB method. Both A260/A230 and A260/A280 ratios were larger than 1.8, suggesting that the isolated DNA had high purity and was free of polysaccharide/polyphenol and protein contamination, respectively (Table 1 and Figure 1).

SRAP-PCR condition

After evaluating DNA template, primer combination concentration and annealing temperate with different treatments, the best SRAP-PCR condition was formed. The PCR reaction mixture (total 25 µl) contained DNA template 20 ng, forward primer 0.4 µM, reverse primer 0.4 µM, 1× Taq MasterMix. The best annealing temperature was 35/50°C. The isolated DNA evaluated by three factors (Figures 2, 3 and 4) were amplified by primer pair Me4/Em4 (Forward: TGAGTCCAAACCGGACC; Reverse: GACTGCGTACGAATTTGA) and an electrophoresis

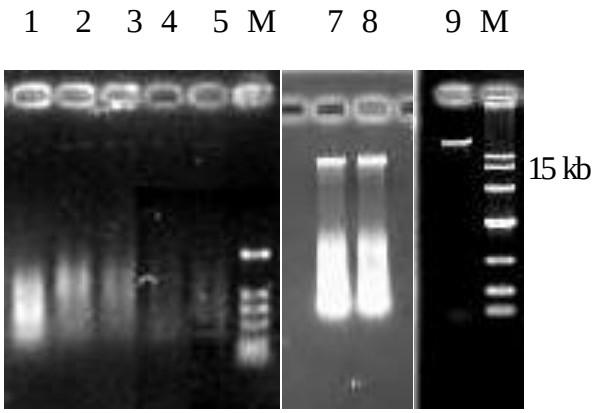

Figure 1. Electrophoresis result of genomic DNA extracted from *P. umbellatus* using different isolation methods. M represented DM 15000 DNA marker; Lane 1-3: DNA extracted by the original CTAB method (Han 2008), treating with adding 35% alcohol (1), 5% NaCl solution (2 and 3), respectively; Lane 4-5: the original CTAB method; Lane 7-9: DNA extracted by the improved CTAB method, containing RNA (7 and 8) and eliminating RNA with RNase treating 30 min.

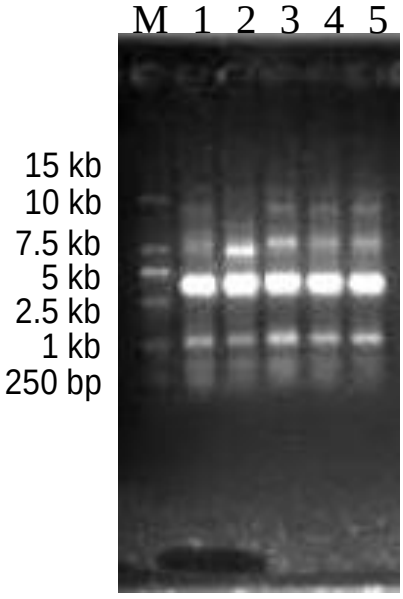

Figure 2. Electrophoresis detection of different DNA template concentrations that extracted from *P. umbellatus*. M represented DM 15000 DNA marker; Lane 1-5: total 25 µl reaction volume containing 10 (1), 15 (2), 20 (3), 25 (4) and 30 ng (5) DNA template, respectively.

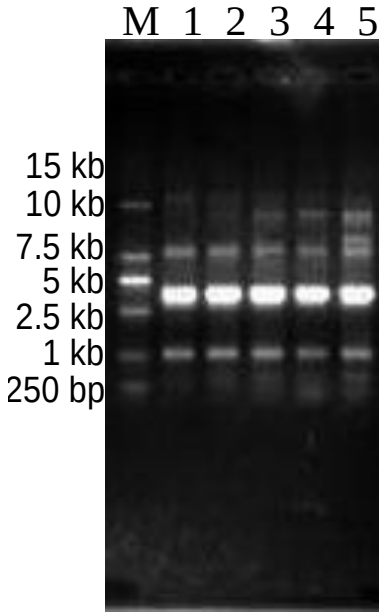

Figure 3. Electrophoresis detection of different primer combination concentrations contained in SRAP-PCR system. M represented DM 15000 DNA marker; Lane 1-5: 0.24 (1), 0.32 (2), 0.40 (3), 0.48 (4) and 0.56 µM (5), respectively.

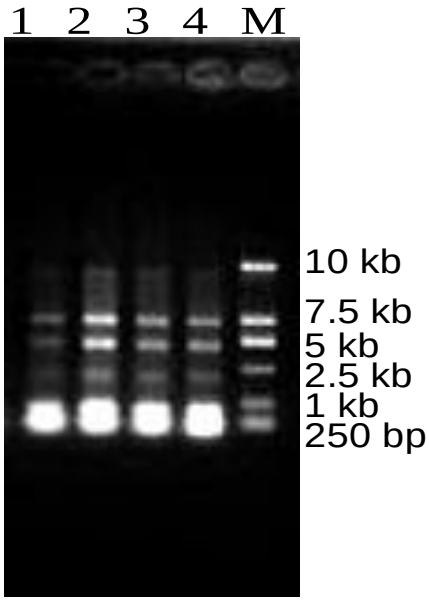

Figure 4. Electrophoresis detection of different annealing temperatures for primer combinations. M represented DM 15000 DNA marker; Lane 1-4: 35/48 (1), 35/50 (2), 32/50 and 30/50 ℃ (4), respectively.

result of all eight strains by using Me6/Em5 and Me6/Em6 primer combinations was displayed in Figure 5. Eighty-one SRAP primer combinations were initially screened by using the best SRAP-PCR condition, of

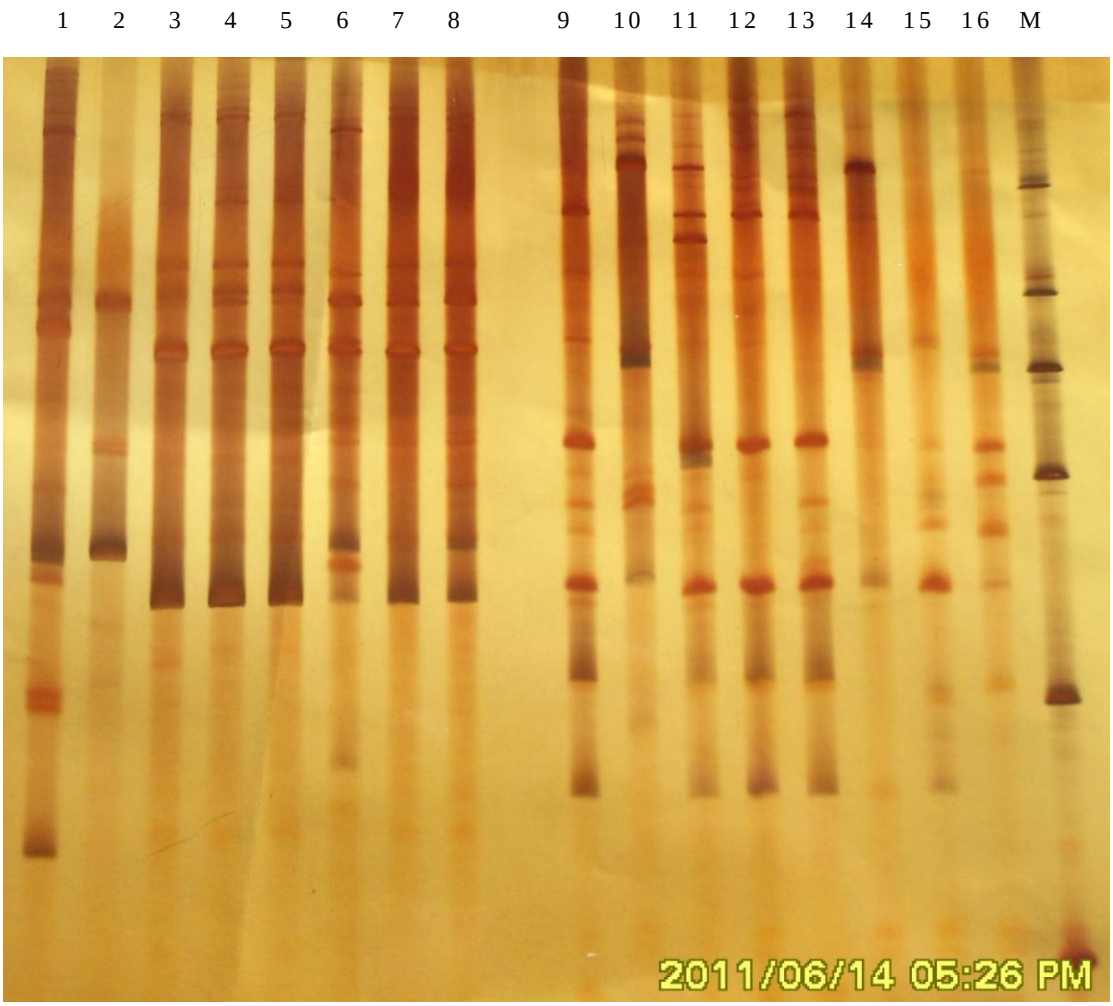

Figure 5. Polyacrylamide gel electrophoresis (PAGE) results of eight *Polyporus umbellatus* strains amplified by both primer combination Me6/Em5 (Lane 1-8) and Me6/em6 (Lane 9-16). M was DNA marker 2000.

which forty-nine was selected that produced consistent and clear polymorphic fragments.

DISCUSSION

Among all molecular markers, SRAP was firstly applied in *P. umbellatus* strains for subsequent resources conservation, sustainable utilization and further research. SRAP aims at the amplification of open reading frames and its markers are more consistent and repeatable than RAPD, and are less-labor-intensive and time-consuming to produce than AFLP and SSR (Li and Quiros, 2001; Ren et al., 2010; Cai et al., 2011). Budak et al. (2004) compared four marker systems in buffalo grass and found the values of revealing genetic diversity power as: SRAP>SSR>ISSR>RAPD. SRAP has been widely applied in genetic diversity analysis, molecular identification, genetic linkage map construction, and gene tagging (Li and Quiros, 2001; Ren et al., 2010; Cai et al., 2011). Our protocol provides the opportunity for the genetic research in Japan or other countries for resources conservation and management in world-wide range, fragments screening of functional gene involved in polysaccharide, and molecular breeding in future, all of which are lacking.

Like other filamentous fungi, *P. umbellatus* has rigid cell walls (Guo and Xu, 1992) which make DNA and RNA isolation more difficult. Although its DNA may be isolated before based on the report of Xing and Guo (2004), the details have not been provided. On the other hand, mycelium of *P. umbellatus* contains more polysaccharide that can severely interfere with DNA, RNA and protein purification (Sánchez-Rodríguez et al., 2008). Our past work showed that the intracellular polysaccharide contents ranged from 23.56 to 64.50 mg/g fresh mycelium weight among seven mycelial strains which sclerotium collected from seven provinces of China (Zhang et al., 2010). Therefore, three additional approaches were performed to eliminate polysaccharide

step by step when extracting DNA from the mycelium: (1) Add RNAiso-mate for plant tissue buffer to the fine powder after ground by using liquid nitrogen; (2) Ethanol (35% volume of extraction buffer) was added to the DNA extraction buffer and was mixed well immediately, making polysaccharide deposit; (3) Half volume of NaCl solution (5%) was added to the mixture of isoamyl alcohol and DNA deposit solution.

Conclusively, the protocol presented here demonstrates isolation of intact high-quality DNA and provides the best SRAP-PCR condition from *P. umbellatus*.

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Review

Amazing bean *"Mucuna pruriens"*: A comprehensive review

Kavitha C.* and Thangamani C.

HC & RI, TNAU, Coimbatore, India.

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Mucuna pruriens **commonly known as cow-age or cowitch or velvet bean or Alkushi, is a medicinal plant traditionally used in Indian medicine belongs to the family Leguminosae.** ***M. pruriens*** **seed is a natural source of the amino acid L-3,4-dihydroxy phenyl alanine (L-DOPA), the direct precursor to the neuro transmitter dopamine which is used widely in the treatment of Parkinson's disease (PD). Serotonin, oxitriptan, nicotine, N,N-DMT, and bufotenine are the other chemicals found in** ***M. pruriens*** **in addition to L-DOPA. According to Ancient Ayurvedic literature** ***Mucuna*** **is used as a potent aphrodisiac, geriatric tonic and vermifuge. It is also used for the treatment of menstruation disorders, constipation, edema, fever, tuberculosis, etc. In addition,** ***Mucuna*** **is also grown as food crop, ornamental plant, living mulch and green manure crop. The reviews summarize the botany, uses, phyto constituents and pharmacological activities of** ***M. pruriens*****.**

Key words: *Mucuna pruriens*, uses, phytoconstituents, L-3,4-dihydroxy phenyl alanine (L-DOPA), phytochemistry, pharmacology.

INTRODUCTION

Hunger and disease are the two extremely vital phenomena which threaten the very survival of mankind in mother earth. To cure himself off the diseases, mankind has tried various methods and strategies. Due to the easy access to the number of plants growing around the dwelling place, the initial empirical attempts were made with plants and plant products. To date, the herbal medicine is still the mainstay of about 75 to 80% of the world population, mainly in the developing countries, for primary health care because of better compatibility with the human body and lesser side effects (Kamboj, 2000). In India, herbal medicine has been used for treatments to cure various diseases (Biswas et al., 2004).

According to WHO (1991), traditional medicine is the synthesis of therapeutic experience of the generations of indigenous systems of medicine. Herbal drugs constitute only those traditional medicines, which primarily use medicinal plant preparations for therapy. The earliest recorded evidence of herbal drugs use in Indian, Chinese, Egyptian, Greek, Roman and Syrian texts dated back to about 5000 years. In India, the earliest mention of the use of medicinal plants was found in the Rig Veda, which is one of the oldest epic and written between 4500 and 1600 BC. Among the medicinal plants, one of the most important medicinal plant and very less exploited is *Mucuna pruriens* (L.) DC. Physicians in ancient India first used *Mucuna* seeds in the treatment of Parkinson's disease (PD) over 4500 years ago. This herb has one of the most fascinating chemical profiles on earth. It is the host of many interesting alkaloids which have profound actions on the human nervous system.

M. pruriens is a tropical legume and is commonly known as velvet bean or cowitch or cowhage or Alkushi. It is one of the most popular medicinal plants of India and is constituent of more than 200 indigenous drug formulations. It is widespread and is found in the plains of

*Corresponding author. E-mail: ck77@tnau.ac.in.

India (Sastry and Kavathekar, 1990; Agharkar, 1991; Singh et al., 1996). The demand for *Mucuna* in Indian as well as in international drug markets increased many fold only after the discovery of the presence of L-3,4-dihydroxy phenyl alanine (L-DOPA), an anti-Parkinson's disease drug in the *Mucuna* seeds (Farooqi, 1999).

BOTANY

The genus *Mucuna* belongs to the family Leguminosae and consists of 100 species of climbing vines and shrubs. The name of the genus is derived from the word mucunã (Umberto, 2000). This genus is found all over the world in the woodlands of tropical areas especially in tropical Africa, India and the Caribbean. *M. pruriens* is a twinning annual that can reach 15 m in length. The plant is almost completely covered with fuzzy hairs when young, but it is almost free of hairs when it grows older (Sahaji, 2011). The leaves are trifoliate, alternate or spiraled, gray-silky beneath; petioles are long and silky, 6.3 to 11.3 cm. Leaflets are membranous, terminal leaflets are smaller, lateral very unequal sized. Flowers are dark purple, white or lavender in colour (6 to 30), pea-like but larger, with distinctive curved petals and occur in drooping racemes. Fruits, longitudinal pods are curved, 4 to 6 seeded and about 10 cm long (Agharkar, 1991) and are densely covered with persistent pale-brown or grey trichomes that cause irritating blisters if they come in contact with skin. The chemical compounds responsible for the itch is a protein, mucunain (Agharkar, 1991) and serotonin. Seeds are shiny black or brown, ovoid and 12 mm long (Sastry and Kavathekar, 1990; Agharkar, 1991; Verma et al., 1993).

PHYTOCONSITUENTS

M. pruriens seeds contain high concentrations of L-DOPA, an unusual non protein amino acid and a direct precursor to the neuro transmitter dopamine, an important brain chemical involved in mood, sexuality and movement. Besides, it also contains some other amino acids, glutathione, lecithin, gallic acid and beta sitosterol. The mature seeds of the plant contain about 3.1 to 6.1% L-DOPA, with trace amounts of 5-hydroxy tryptamine (serotonin), nicotine, dimethyl tryptamine (DMT), bufotenine, 5-MeO-DMT and beta-carboline. The leaves contain about 0.5% L-DOPA, 0.006% dimethyl tryptamine and 0.0025% 5-MeO-DMT (Erowid, 2002).

TRADITIONAL USES

All parts of *Mucuna* possess valuable medicinal properties (Caius, 1989). It is used against a wide range of disorders, such as urinary tract, neurological and menstruation disorders, constipation, edema, fever, tuberculosis, ulcers, PD (Katzenschlager et al., 2004) and helminthiases like elephantiasis (Oudhia, 2002). Traditionally, the powdered seeds of *M. pruriens* was found to increase the general mating behavior and thereby sexual activity in rats (Amin et al., 1996).

Roots, according to the Ayurveda, are bitter, thermogenic, anthelmintic, diuretic, emollient, stimulant, aphrodisiac, purgative, febrifuge, and tonic. It is considered useful to relieve constipation, nephropathy, dysmenorrhoea, amenorrhoea, elephantiasis, dropsy, neuropathy, ulcers, helminthiasis, fever and delirum (Warrier et al., 1996).

Leaves are popular potherbs and are used as a fodder crop. Leaves are useful in ulcers, inflammation, cephalagia and general debility. Dried leaves of *M. pruriens* are sometimes smoked. The pods are covered with coarse hairs, trichomes which cause itching, blisters and dermatitis. Pods are also used as vegetable. Pod hairs are used as anthelmintic. Hairs mixed with honey are used as vermifuge. An ointment prepared with hairs act as a local stimulant and mild vesicant (Sastry and Kavathekar, 1990).

The plant and its extracts are used in tribal communities as a toxin antagonist for various snakebites. Seed is a source of minerals (Singh et al., 1995). According to Ayurveda, seeds are astringent, laxative, astringent, laxative, anthelmintic, aphrodisiac, alexipharmic and tonic. Seeds are found to have antidepressant properties in cases of depressive neurosis when consumed (Oudhia, 2002) and formulations of the seed powder have shown promise in the management and treatment of PD (Chamakura, 1994).

OTHER USES

Beside medicinal properties, in many parts of the world, *M. pruriens* is used as an important forage, fallow and green manure crop. Since the plant is a legume, it fixes nitrogen and fertilizes soil. *M. pruriens* is a wide spread fodder plant in the tropics. The whole plant is fed to animals as silage, dried hay or dried seeds. *M. pruriens* silage contains 11 to 23% crude protein, 35 to 40% crude fiber and the dried beans contain 20 to 35% crude protein. It is also used as a biological control for problematic *Imperata cylindrica* grass in Benin and Vietnam (Kavitha and Vadivel, 2008).

The genus is also used as living mulch for tropical areas and it increases phosphorus availability after application of rock phosphate (Vanlauwe et al., 2000). *M. pruriens* was used in Native American milpa agriculture. *Mucuna* is also used as a food crop, e.g. in Eastern Nigeria, although the L-DOPA content makes it less desirable. The plant is to be processed before it can be eaten, that is, the leaves must be soaked to leach out the L-DOPA. The seeds are also cracked open and soaked

before eaten. If consumed in large quantities as food, unprocessed *M. pruriens* is toxic to non-ruminant mammals, including humans (Oudhia, 2002; Diallo and Berhe, 2003).

CULTIVATION

Mucuna is grown as kharif crop in India. Seeds are sown at rate of 50 kg/ha with plant spacing of 60 × 60 cm. Although, no named cultivar of *Mucuna* is available, locally available seeds possess good viability and higher germination (Oudhia, 2001). Plant needs support for growth. Increase in yield upto 25% and reduction in pest infestation is obtained by providing support. Normally, flowering begins in 45 to 50 days after sowing (Oudhia and Tripathi, 2001).

Thomas and Palaniappan (1998a, b) found that application of 50 kg P_2O_5 ha^{-1} significantly increased the growth, yield components and seed yield of velvet bean. Kumwenda and Gilbert (1998) stated that *M. pruriens* had the highest biomass production averaging 7.3 t ha^{-1} with P_2O_5 application. The nutrient omission trial in *Mucuna* showed that when N and P were absent in the complete fertilizer treatment, biomass production decreased significantly on an average by 69% (N) and 33% (P) (Houngnandan et al*.,* 2001). Philip et al*.* (2001) found that dry matter production under 30 and 45 kg P_2O_5 ha^{-1} application was at par in *M. bracteata.* Better growth of the plant, increased number of leaves and longer duration of leaves due to delayed senescence were observed when *M. pruriens* plants were provided with both organic (cocopeat at 5 t/ha and farm yard manure at 12.5 t/ha) and inorganic forms (NPK 40:30:30 kg/ha) of nutrition (Kavitha and Vadivel, 2006a).

Biomass yield of *Mucuna* varies directly with length of growing season and soil fertility conditions. High biomass accumulation (10 t ha^{-1}) was observed in areas of longer growing season. Varietal characteristics were also observed to influence the rate of dry matter production (IITA, 1997). Becker and Johnson (1998) reported that soil phosphorus is an important factor in *Mucuna* biomass accumulation, as legumes require phosphorus for growth and nitrogen fixation. The integrated nutrient combination involving organic manures (cocopeat at 5 t/ha and farmyard manure at 12.5 t/ha) and inorganic fertilizers (NPK 40:30:30 kg/ha) and harvesting at dry pod stage showed a greater degree of positive influence on dry matter production (Kavitha and Vadivel, 2008).

According to Kay (1979) seed yield in *Mucuna* ranged from 700 to 1100 kg ha^{-1} in India, 1700 to 2200 kg ha^{-1} in USA and 600 kg ha^{-1} in Australia. Humphreys and Riveros (1986) reported that staking is generally recommended for improving quantity and quality of *Mucuna* seed production. The globular or reniform seeds are usually coloured black, white, creamy yellow or may be mottled and are about 4 to 8 seeds per pod. Hundred seed weight varied from 25 to 110 g (Buckles, 1995). Chadha (1995) reported that seed yield varied between irrigated and rainfed crop. In a rainfed crop without staking seed yield of 1500 to 1750 kg ha^{-1} and with staking, yield of 3000 to 3750 kg ha^{-1} were obtained. Yield upto 5000 kg/ha have been recorded from well managed irrigated crop provided with stakes (Singh et al., 1995; Farooqi et al., 1999). Kavitha and Vadivel (2006b) reported that the integrated nutrient combination involving organic form of manures (cocopeat at 5 t/ha and farmyard manure at 12.5 t/ha) and inorganic fertilizers (NPK 40:30:30 kg/ha) resulted in high seed yield.

L-DOPA

Dymock and Warden (1980) reported that the presence of L-DOPA, a precursor of dopamine in the seeds of *M. pruriens* made the plant valuable in the treatment of PD. *M. pruriens* is used in Ayurvedic medicine to treat diseases of the central nervous system and geriatric disorders (Mahajani et al., 1996). Climatic factors are observed to have no direct effect on L-DOPA content in *M. pruriens* while plants supported by stakes have higher L-DOPA content (Pieris and Janz, 1980). Fujii et al. (1992) stated that L-DOPA is present at about 1% by fresh weight in leaves and roots of *M. pruriens*. Sunitha (1996) reported that there was no significant difference in the content of L-DOPA when *M. pruriens* was grown under shade or open conditions. Prakash and Tiwari (1999) investigated the variation of L-DOPA contents in different parts of *Mucuna* species, namely, fully matured seeds 3.6 to 4.2%, pod-pericarp 0.14 to 0.22%, leaves 0.17 to 0.35%, stems 0.19 to 0.31% and roots 0.12 to 0.16% and the highest amount of L-DOPA was found in half mature seeds. Vadivel and Janardhanan (2000) reported that the content of L-DOPA in the seeds of different accessions ranged from 7.62 to 8.37%. Ramaswamy (1957) reported on the isolation of L-DOPA (1.5% on dry weight basis) from *Mucuna* seeds. Siddhuraju and Becker (2001) developed a high performance liquid chromatographic assay for the extraction and quantitative determination of L-DOPA in *M. pruriens* var *utilis* seeds. Yang et al. (2001) reported that high performance liquid chromatography (HPLC) analysis revealed L-DOPA concentrations ranged from 3.9 to 6.2% in *Mucuna* seeds.

In vitro production

To accommodate the huge demand for L-DOPA, *in vitro* production of the drug using cell cultures, is now practiced extensively. The presence of L-DOPA in callus and cell suspension cultures of *M. pruriens* has been reported (Brain, 1979; Obata-Sasamata and Komamine, 1983). Huizing et al. (1985) demonstrated the presence

of L-DOPA in the cell suspension cultures of *M. pruriens* by means of thin layer chromatography (TLC) and HPLC. Wichers et al. (1985) reported that addition of 2,4–D to the cell suspension culture medium of *M. pruriens* suppressed L-DOPA production. Huizing (1986) reported that the synthesis of L-DOPA was upto 90% from *M. pruriens* cultures. Wichers et al. (1993) detected the presence of dopamine in the cell suspension cultures of *M. pruriens*. Chattopadhyay et al. (1994) reported that discernibly higher production of L-DOPA, from *M. pruriens* f. *pruriens* cell cultures was obtained in comparison to single stage culture. Huang et al. (1995) concluded that the high yields of L-DOPA was associated with the precise control of pH, adequate Indole-3-Acetic Acid (IAA) concentration and a yellowish or brown cell colour which reflected melanin production in cell line culture of *Stizolobium hassjoo*. Huang and Chen (1998) reported that efficient production of L-DOPA is possible in *Stizolobium hassjoo* cell culture in a two stage configuration. Murashige and Skoog (MS) medium with 2 mg/L 2,4-D was found to be the best medium for inducing callus in various explants, namely, stem bits, leaf bits and root bits and L-DOPA accumulation was more in cell suspension culture in liquid MS medium with 4% sucrose + 1 mg/L IAA + 1 mg/L BA (Kavitha and Vadivel, 2005).

PHARMACOLOGY

Anti-Parkinson's activity

The clinical syndrome of Parkinsonism was identified in ancient India even before the period of Christ and was treated methodically. According to "Bhasava rajyam" the Parkinsonism was treated by the administration of powdered seed of *M. pruriens* containing 4 to 6% of levodopa (Ovallath and Deepa, 2013).

Hussian and Manyam (1997) indicated that for the dose, *M. pruriens* showed twice the anti-Parkinsonian activity of synthetic L-DOPA. In a clinical study, Nagashayana et al. (2000) revealed the contribution of L-DOPA in the recovery of PD followed by Ayurveda medication. Katzenschlager et al. (2004) revealed that 30 g *Mucuna* seed powder preparation has considerable faster action in treating PD patients than conventional standard drugs, namely, Levodopa or Carbidopa and suggested that natural source of L-DOPA might possess advantages over conventional drugs in long term management of PD.

Antiglycaemic effect

Using a combination of chromatographic and NMR techniques, the presence of D-chiro-inositol and its two galacto-derivatives having antiglycaemic effect was demonstrated in *M. pruriens* seeds (Donati et al., 2005).

Hypoglycemic activity

The hypoglycemic effect of the aqueous extract of the seeds of *M. pruriens* was investigated in normal, glucose load conditions and streptozotocin (STZ)-induced diabetic rats. In normal and STZ diabetic rats, the aqueous extract of the seeds of *M. pruriens* (100 and 200 mg/kg body weight) significantly reduced the blood glucose levels 2 h after oral administration of seed extract. It also significantly lowered the blood glucose in STZ diabetic rats after 21 days of daily oral administration of the extract. Thus, it was clearly depicted that *M. pruriens* could be a source of hypoglycemic compounds (Bhaskar et al., 2008).

Antioxidant activity

In vitro assays indicated that a whole plant of ethyl acetate and methanolic extract of *M. pruriens*, containing large amounts of phenolic compounds, exhibited high antioxidant and free radical scavenging activities. These plant extracts served as a significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses (Satheesh et al., 2010; Kumar and Muthu, 2010).

Antivenom activity

Research on its effects against *Naja* species (Tan et al., 2009) has shown it has potential use in the prophylactic treatment of snakebites. Aqueous extracts of *M. pruriens* seeds were tested for their activity on various pharmacological effects like lethality, phospholipase activity, edema forming activity, fibrinolytic activity and haemorrhagic activity of cobra and krait venoms. About 0.16 and 0.19 mg of *M. pruriens* seed extracts were able to completely neutralize the lethal activity of $2LD_{50}$ of cobra and krait venom, respectively, thus suggesting that aqueous extracts of *M. pruriens* seeds possess compounds, which inhibit the activity of cobra and krait venoms (Meenatchisundaram and Michael, 2010). According to Fung and Tan (2012), rats pretreated with *M. pruriens* seed extract showed protective effect against the lethal and cardiovascular depressant effects of *Naja sputatrix* venoms by neutralization of the venom toxins.

Aphrodisiac activity

Shukla and Mahdi (2010) demonstrated that oral administration of 5 g of *Mucuna* seed powder once in a day for men with decreased sperm count and motility ameliorated psychological stress and seminal plasma liquid peroxide levels along with improved sperm count and motility. The study also concluded that *M. pruriens*

not only reactivates the anti oxidant defense mechanism, but also helps in the management of stress and improves semen quality.

Antimicrobial activity

M. pruriens is also used for antimicrobial properties for extracting plant metabolites against plant pathogenic bacteria and fungi. The methanolic extract showed high antibacterial activity against *Erwinia carotovora*, *Pseudomonas syringae*, *Pseudomonas marginalis*, *Pseudomonas acruginosa*, *Xanthomonas campestris* and high anti fungal activity against *Curvularia lunata*, *Fusarium oxysporum*, *Pencillium expansum*, *Rhizoctonia solani*, *Tiarosporella phaseolina*, *Ustilago pomaydis* (Rayavarapu and Kaladhar, 2011).

CONCLUSION

M. pruriens, the fascinating herb is abided with manifold uses. All parts of the plant are being used in pharmacological preparation. Hence, due importance should be focused on this particular plant and more research has to be carried out to exploit the total potential of the crop in the field of pharmacology.

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