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Molecular Detection of *blaZ* and *mecA* Genes and Study of Antibiotic Resistance Pattern in Clinical Isolates of *Staphylococcus aureus* from Bovine Mastitis in Coastal Andhra Pradesh

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Abstract

Introduction: *Staphylococcus aureus* is known to cause sub clinical and clinical intra mammary infections like mastitis in milch animals. *S. aureus* demonstrates a distinctive ability to quickly develop a resistance mechanism, starting with penicillin, until against even the most recent, linezolid and daptomycin.

Materials and Methods: In the present study, 100 milk samples from mastitis of buffaloes in coastal Andhra Pradesh were collected. These isolates were characterized initially by biochemical tests like Mannitol fermention, Catalase test, Coagulase test, Spot Oxidase test, Voges – Proskauer test and the Haemolytic activity on 5 % Sheep Blood agar. Provisionally confirmed isolates of *S. aureus* were further processed for molecular detection of *S. aureus* with species specific oligonucleotide primers Staur 4 and Staur 6. The sensitivity of confirmed isolates of *S. aureus* to different antimicrobials was tested against 10 antimicrobials by Kirby-Bauer disc diffusion method. The presence of antibiotic resistance genes like b-lactamase (*blaZ*) and methicillin resistance (*mecA*) were screened by PCR using specific primers.

Result: Out of 100 isolates, 69 samples were provisionally positive for *S.aureus* by biochemical methods and 34 isolates (34.0%) by species specific PCR. High resistance was recorded to Ceftriaxone+Tazobactum, Oxacillin and Amoxycillin (100%), followed by Ceftriaxone+ Sulbactum (93.55%) and Methicillin (83.87%), Penicillin (80.65%), Ampicillin (70.97%) and Ceftriaxone (38.71%). Low resistance was observed for Cefoxitin(6.47%) and Amoxycillin+ Clavulanic acid(0). A high frequency of *mecA* (61.29%) and *blaZ* (45.16%) antibiotic resistance genes was found in *S. aureus* isolates. Both *mecA* and *blaZ* genes were present in 22.58% of *S. aureus* isolates.

Conclusion: The results show that the organisms are acquiring resistance against commonly employed antimicrobials to treat mastitis by acquiring antibiotic resistance genes at an alarmingly fast rate.

Key Words: *Staphylococcus aureus*, antibiotic resistance, Mastitis, *blaZ*, *mecA*.

Introduction

In India, losses due to mastitis in dairy animals are estimated to be about Rs. 6053.21 crore per annum (1). Approximately 8% to the GDP of Indian economy is contributed by dairy industry. But the onslaught of diseases likes mastitis, not only in India but globally, leads to enormous losses to the dairy sector. As a consequence, this big economic sector is suffering several setbacks including bacterial

pathogens, requiring scientific interventions urgently needed to curb the menace of such emerging diseases of livestock in the country. One of such disease is mastitis, causing serious wastage and undesirable milk quality in dairy development of tropics. Subclinical mastitis is common in India, varying from 10–50% in cows and 5–20% in buffaloes than the clinical mastitis (1–10%) (2). *Staphylococcus aureus* is one of the most important causative agents in sub clinical and clinical intra mammary infections like mastitis in milch animals. The Indian dairy industry is facing economic losses by mastitis due to its adverse impact on milk production of cows and buffaloes (2 & 3).

The ability of *S. aureus* to develop or acquire strategies which provide resistance to different antimicrobials is an additional approach in the impressive arsenal of this pathogen. Antibiotic therapy is a significant tool used in the scheme of mastitis control. The development of resistance among different bacterial strains is because of misuse or intensive use of antibiotics for treatments (4). One of the diagnostic tools accessible to practitioners to assist with the selection of a suitable treatment is the *in vitro* testing of isolates against a representative panel of antimicrobial drugs. The susceptibility pattern of the implicated strain enables crucial action to be taken by the veterinarian in terms of treatment (5). This avoids the needless application of ineffective antimicrobials and prevents unnecessary costs from being incurred.

Extensive and inadvertent use of antimicrobials both in human and in veterinary medicine is the key reason for emergence of resistant strains of *S. aureus* (6). Emergence of MRSA (Methicillin Resistant *S.aureus*) and VRSA (Vancomycin Resistant *S.aureus*) have been reported in livestock in past (7-14). There is an enormous increase and emergence of *S. aureus* strains resistance to the antibiotic methicillin (MRSA strains) and also to the beta lactam antibiotics like penicillin and its derivatives over the past few decades. The antimicrobials generally used for the treatments of infections caused by *S. aureus* were penicillin and its derivatives, including methicillin (15). The presence of *mecA* and *blaZ* genes in a particular strain is responsible to the intrinsic resistance to these antimicrobials. Due to the development of antibiotic resistance in *S. aureus*, conventional antibiotic treatment which was used frequently at field level is not satisfactory for preventing the establishment of chronic infection or in eliminating existing disease. There are three different types of MRSA namely Hospital Acquired MRSA (HA-MRSA), Community Acquired MRSA (CA-MRSA) and Livestock Associated MRSA (LA-MRSA) (16). As the treatment options for this highly zoonotic MRSA are limited, these infections have gained importance. There is a drastic rise in number of reports of MRSA in domestic dairy animals (7-11). Cross-infection of certain strains of MRSA between humans and animals, were also reported (17). It was reported in several parts of the world that MRSA causes life threatening sepsis, endocarditis and osteomyelitis in human beings. Animals can thus act as potential source of MRSA infection to in-contact human beings (18). Thus the development of multidrug resistance in MRSA possesses a serious public health concern too.

In this context, the present investigation has been carried out with an aim to know the antibiotic sensitivity pattern of *Staphylococcus aureus* isolates with special reference to MRSA. A total of 100 mastitis milk samples were collected from three different districts from Coastal Andhra Pradesh, namely Krishna, Guntur and East Godavari and were evaluated for the presence of *S. aureus.* The *S. aureus* isolates were tested for their susceptibility to a panel of antimicrobial agents which are frequently used for the treatment of *S. aureus* in human beings. The present study enables to assess the potential zoonotic threat of *S. aureus* from animal origin which are resistant to multiple drugs commonly employed in human antimicrobial therapy.

Materials and Methods

***Sample Collection*:** A total of 100 milk samples were collected from buffaloes affected with

clinical mastitis. Approximately15 - 20 ml of milk sample from mastitis infected buffalo was collected in sterile vial after cleaning the teat orifice with 70% ethyl alcohol and after discarding the first few streams of milk. Collected samples were screened by California Mastitis Test. They were further processed for the isolation of *S. aureus*.

Isolation of S. aureus: The clinical milk samples were inoculated in Tryptic Soy Broth (TSB) (M/S Oxoid, UK) for enrichment and further subcultured on Mannitol Salt Agar (MSA) (M/S Oxoid, UK) as it is a selective and differential medium for *S.aureus* (19). The inoculated plates were incubated at 37^{0}C for 24hrs and the suspected colonies, being gram positive cocci by Gram's staining were picked up and processed for biochemical characterization.

***Biochemical Characterization*:** The suspected *S. aureus* isolates were characterized by Catalase test, Oxidase test, Voges- Proskauer test and Coagulase test. *Staphylococci* produce coagulase which coagulates the rabbit plasma identifying them as pathogenic isolates (20). Haemolysis pattern was tested on 5% sheep blood agar medium (16 & 21).

The provisionally confirmed *S.aureus* isolates were further confirmed By PCR test using species specific primers.

***DNA Extraction*:** The tryptic soy broth (TSB) was inoculated with biochemically characterized *S. aureus* and incubated for 18 hrs at 37°C for enrichment. From this, 2 ml of enriched culture was taken and centrifuged at 5,000 rpm for 10 min to pellet the bacterial cell mass. The DNA was isolated as described by Aravindakshan *et al*. (22) with suitable modifications (23). DNA pellet was consequently re-suspended in 40ìl of sterile milli-Q water and kept at -80°C for further use in PCR

***Molecular Characterization*:** Molecular characterization of *S. aureus* was done using species specific primers by PCR in Master cycle rep gradient thermal cycler (M/s Eppendorff, Germany). The forward and reverse primer sequences which were used in PCR test were 5'ACGGAGTTACAAAGGACGAC 3' and 5'AGCTCAGCCTTAACGAGTAC 3' respectively (24) synthesized at Bioserve Biotechnologies (India) Pvt. Limited, India. (Table 1)

PCR Amplification: The PCR reaction was performed in a 10ìl reaction mixture consisting

Table 1. Oligonucliotide primers and PCR test conditions for *S.aureus* and detection of antibiotic resistant genes for *blaZ* and *mecA*

Gene	Primers	Product size	Sequence (5'- 3')	Denaturation	Annealing	Extension
S. areus 23s rRNA	Staur 4 Staur 6	1250bp	ACGGAGTTACAAAGGACGAC AGCTCAGCCTTAACGAGTAC	94^{0}C /45sec	64^{o}C/60sec	72^{o}C/2min
BlaZ	*blaZ* F *blaZ* R	517	AAGAGATTTGCCTATGCTTC GCTTGACCACTTTTATCAGC *(Vesterholm-Nielsen* ***et al.,*** *1999)*	94^{0}C/4min	94^{0}C /60sec	55^{o}C/60sec
MecA	mecA F mecA R	162	TCCAGATTACAACTTCACCAGG CCACTTCATATCTTGTAACG ***(****Stegger et al., 2012****)***	94^{0}C/45sec	50^{0}C/30sec	72^{0}C/30sec

F= Forward primer R=Reverse Primer

of 5ìl Go Taq Green Master Mix (Promega, USA), 0.25ìl of forward and reverse primer each, 0.5ìl of DNA template to which 4.0ìl of distilled water was added. The master mix contained 2X Green Go Taq Reaction Buffer (pH 8.5), 3 mM $MgCl_2$ and 400µM of each dNTP. The PCR conditions starts with initial denaturation and subsequent denaturation carried out at 94°C for 2 min and 45 sec respectively, followed by annealing at 64°C for 60 sec, continued by extension and final extension at 72°C for 2 min and 10 min respectively. A total of 35 PCR cycles were run with the same conditions.

The amplified PCR products were analyzed by electrophoresis on a 1.7% agarose gel stained with 0.5ìg of ethidium bromide / ml in Tris Borate EDTA (TBE) buffer. Electrophoresis was performed at 90 V for 120 min in submarine gel electrophoresis unit (M/s Atto Corporation, Japan) and finally the PCR products were visualized in InGenius Gel Documentation System, (M/s Syngene, U.K) along with a ProxiO 100bp DNA ladder (M/s BioLit, SRL,India).

***Antimicrobial Susceptibility Testing by Disc Diffusion Assay*:** All the positive *S.aureus* isolates were tested for susceptibility for the following 10 antimicrobial agents: amoxicillin (30 mcg), amoxicillin + clavulanic acid (30/15 mcg), ceftriaxone (30mcg), ceftriaxone + tazobactam (30/10 mcg), ceftriaxone + sulbactam (30/15 mcg), oxacillin (5 mcg), penicillin (10 mcg), ampicillin (10 mcg), methicillin (5 mcg), and cefoxitin (10 mcg). The disc diffusion assay and zone interpretation of each antimicrobial agent was done according to Clinical and Laboratory Standards Institute, 2012 (CLSI, 2012).

***Molecular Detection of blaZ and mecA Genes by PCR*:** The PCR assays were performed for the detection of antibiotic resistance genes *mecA* responsible for methicillin resistance and *blaZ* gene responsible for penicillin resistance. The primer sequences and PCR conditions were given in the table 1. PCR was run for 35 cycles with initial denaturation at 94ºC for 4 min and final elongation at 72ºC for 10min for *blaZ* oligonucleotide primer sets. And 35 cycles with initial denaturation at 94ºC for 5 min and final elongation at 72ºC for 10min for *blaZ* oligonucleotide primer sets.

Results

The study has been conducted by using 100 mastitis milk samples were collected and tested for the presence of *S. aureus*. Out of 100 milk samples 40 cases were clinical mastitis whereas 60 were subclinical mastitis after testing by California Mastitis Test.

Isolation of S. aureus: Out of 100 samples collected, 69 (69.0%) isolates were mannitol fermenting when cultured on Mannitol Salt Agar (MSA) medium and all the isolates were gram positive cocci.

***Biochemical Characterization*:** All the 69 isolates were catalase, Vogus Proskauer and coagulase positive whereas oxidase negative.

Haemolysis: Out of 69 isolates 20 isolates were α haemolytic, 10 isolates were â haemolytic and 5 isolates shown both á and â haemolysis on 5% sheep blood agar medium. Remaining 34 isolates were non haemolytic.

***Detection of S.aureus by PCR*:** 34 isolates out of 69 were confirmed as *S. aureus* by PCR test (Fig 1). These 34 isolates were further tested to study the antimicrobial resistance pattern to different antimicrobial agents.

***Antimicrobial Susceptibility Testing by Disc Diffusion Assay*:** Antimicrobial Susceptibility Testing to the molecular confirmed *S. aureus* isolates was performed by Disc Diffusion Assay and the results are shown in Fig 2. A total of 10 different antimicrobials were tested against 34 S. aureus isolates. Maximum resistance was recorded to oxacillin, ceftriaxone and tazobactam (100%), followed by ceftriaxone+ sulbactam (93.55%) and methicillin (83.87%). These were followed by Penicillin (80.65%), Ampicillin (70.97%) and Ceftriaxone (38.70%). Minimum resistance was found to Cefoxitin (6.47%) and Amoxycillin + Clavulanic Acid (0.0%). The results show that the organisms are acquiring resistance

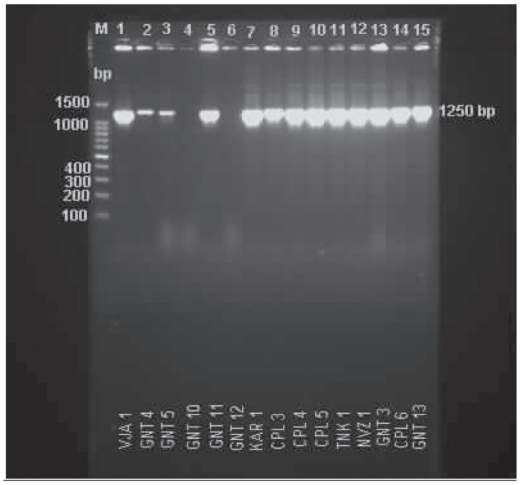

Fig. 1. Gel documentation picture – species conformation of isolates by specific primers

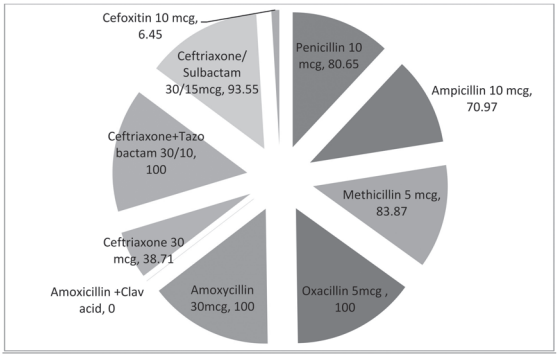

Fig 2. Resistance pattern of isolated organisms- antibiotic sensitivity test

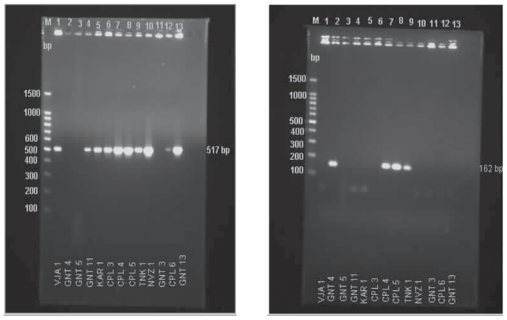

Fig 3. Gel documentation picture showing the presence of antibiotic resistance genes blaZ and mecA in isolated organisms

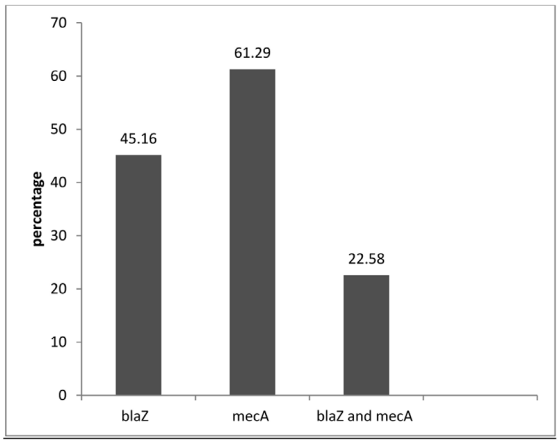

Fig. 4. Distribution of Antibiotic Resistant Genes mecA and blaZ in S. aureus isolates

against commonly employed antimicrobials to treat mastitis.

***Detection of mecA and blaZ in S.aureus Isolates*:** Total of 34 isolates were screened for the presence of antibiotic resistance genes namely, *blaZ* and *mecA*. Out of which in 14 (45.16%) isolates *blaZ* gene was present and in 19 (61.29 %) isolates *mecA* gene was present. Besides, in 7 (22.58%) isolates both *blaZ* and *mecA* genes were present (Fig 4).

Discussion

Bovine mastitis is a significant intramammary infection in animals and *S. aureus* is one of the major pathogen in bovine mastitis. It causes serious loss in agriculture and animal husbandry. *S. aureus* is known for its dynamic virulent characteristics (25). The distribution of α, β-haemolysin and coagulase was in agreement with earlier findings (25,26 & 23). In another study done by us, it has been found that 68% of the samples were positive for *S.aureus* (27) of bovine mastitis in coastal Andhra Pradesh whereas in the present investigation only 31% were positive for *S. aureus*. Similar to our findings, other workers from India like Chavan *et al*. (28) in Hissar found prevalence of *S. aureus* in Mastitis cases 38.66% coagulase positive and

29.33% of coagulase negative *S. aureus*. Sharma *et al*, (29) and Roychoudhury and Dutta (30) have also reported many positive cases of *S. aureus*. Many workers have found *S. aureus* to be more prevalent than other species of the same genus (31, 32, 33 & 34) in mastitis. However, very few systematic reports were available for bovine mastitis problem in coastal Andhra Pradesh where dairy industry is one the major source of income for farmers.

Therefore in the present study we have tried to address this problem. A total of 100 *S. aureus* isolates from milk samples were tested for antibiotic resistance to obtain crucial information regarding the potential threat of antibacterial resistance in animal diseases and its possible zoonotic potential. A huge number of isolates were observed to demonstrate resistance to multiple antimicrobials. Frequent and long-term use of a particular antibiotic in a specific region creates a selection pressure in the organisms, resulting in development of resistance in bacteria.(23,35 &36) In the present investigation, a very high level of resistance was recorded to oxacillin, ceftriaxone and tazobactam (100%), followed by ceftriaxone+ sulbactam (93.55%) and methicillin (83.87%). These were followed by Penicillin (80.65%), Ampicillin (70.97%) and Ceftriaxone (38.70%). Minimum resistance was found to Cefoxitin (6.47%) and Amoxycillin + Clavulanic Acid (0.0%). Followed by Cefoxitin(3.3%), Ceftriaxone and Linezolid (9.68%), Streptomycin (12.90%), Neomycin (25.81%) and Enrofloxacin (29.03%). This result is alarming as the organisms are acquiring resistance against commonly employed antimicrobials for Gram positive bacteria used in human beings. The percentage of penicillin-G resistant isolates (80.65%) in this study was higher than those reported in American herds and European herds (37, 38 & 39). There was a higher prevalence of MRSA (48.39%) as compared with those in similar reports given by Moon *et al*, Kumar and Van den Eede *et al* (36, 23 & 40). Moreover, the multidrug resistance proportion was higher in MRSA than in MSSA isolates for various antimicrobials (41 & 23). Unlike the findings of Pankaj *et al*. (42), in the present study *S. aureus* isolates (100%) were resistant to ceftriaxone. Studies conducted by several workers (43,28,30 & 44) have showed increased resistance towards different traditional and newly introduced antimicrobials. In support to these studies, the antibiogram obtained in the current study indicated higher resistance towards newer and older antimicrobials. This proves that *S. aureus* demonstrates a distinctive ability to quickly respond to newer antimicrobials with the development of an appropriate resistance mechanism. The exact mechanism of development of resistance requires a thorough investigation since it creates an alarming situation of non-responsiveness of antibiotic and transmission of resistance across other genera.

Further the positive *S. aureus* isolates were screened for the presence of antibiotic resistant genes like *blaZ* which is responsible for penicillin resistance and *mecA* gene responsible for methicillin resistance. In the present investigation the percentage positive for *mecA* was 61.29%, whereas in the investigation carried by Memon *et al*. (45) there was no presence of *mecA* gene in *S. aureus* isolates from bovine mastitis. Lee (46) isolated 525 *S. aureus* isolates, out of which 19 (3.61) were positive for *mecA* gene from bovine mastitis cases. In Finland out of 135 isolates only one isolate of *S. aureus* from bovine mastitis was *mecA* positive (47) and in another study carried in West Bengal only 18.42% was positive for the presence of *mecA* gene in *S. aureus* from bovine mastitis (48). In Tamilnadu (49), the percentage positivity for *mecA* was 10.34. The present study is clear evidence that is a high risk of emergence of MRSA in India with special reference to Coastal Andhra Pradesh. This may probably due to improper use of antimicrobials by unqualified people, which is prevailing in this area, though we don't have conclusive treatment history of individual animals that are tested. Unlike to the results of Memon *et al.* (45) where they got 82% 0f *blaZ* positive in the present work the percentage positive of *blaZ*

gene was 45.16%, whereas in Tamilnadu it was 10.34%.

Depending upon the type of organisms and use of antimicrobials in a particular region antibiotic resistance patterns vary among different farms, regions, states and countries. Prudent use of antimicrobials in the dairy animal is important, necessary and worthwhile. Therefore, antimicrobial sensitivity test is recommended before institution of treatment, so that injudicious antibiotic usage and thus the development of resistance is prevented. Moreover, prophylactic management measures against mastitis, rather than therapeutic management using antimicrobials has to be encouraged in dairy industry. The information obtained from the present study will be of helpful to the dairy industry and veterinarians and zoonotic disease experts which will be useful not only in prioritizing mastitis control efforts but in zoonotic transmission control as well.

Conclusion

Milk samples from 100 mastitic buffaloes in four districts of Andhra Pradesh were collected and antibiotic sensitivity test was done to assess the resistance pattern. It has been found that the organisms possess multiple antibacterial resistance, characterized by b-lactamase (45.16%) and methicillin resistance genes (61.29%). Since the farmers live in close proximity to the animals, it can be of a significant public health concern.

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Pathogenic Variability of *Fusarium fujikuroi* Causing Bakanae Disease of Rice (*Oryza sativa* L.)

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Abstract

Bakanae or foot rot disease is one of the newly emerged diseases of rice in Kashmir. An extensive survey conducted during 2014 in two districts of south Kashmir *viz.,* Anantnag and Kulgam, revealed the prevalence of the disease in all the surveyed areas with varied levels of incidence which ranged from 0.6 per cent to 19.3 per cent. The fungus inciting the disease was identified as *Fusarium fujikuroi* Nirenberg [teleomorph*: Gibberella fujikuroi* (Sawada) Ito] on the basis of its morphological and pathological characteristics. Pathogenic variability study of 20 *F. fujikuroi* isolates collected from 17 diverse locations, on a set of seven putative rice differential lines, revealed the existence of variability in terms of pathogenicity of the isolates. The twenty test isolates discern into six pathogenic groups on the basis of similarity in reaction pattern of the differential lines against the test isolates. Two isolates from Block Larkipora (Ff13 and Ff15) shared a single group *i.e,* Group I and 5 isolates (Ff1, Ff2, Ff3, Ff4 and Ff5) from Larnoo block accommodated in Group II, Similarly, three isolates (Ff10, Ff12 and Ff17) from Block Qaimoh and three isolates (Ff6, Ff7 and Ff8) from block D.H.Pora shared group V and VI, respectively. Group III accommodated three isolates, 2 from Achabal block (Ff9 and Ff11) and 1 from Pahloo block (Ff16) and Group IV accommodated 4 isolates, 2 (Ff14 and Ff20) from Pahloo block, 1 (Ff18) from Achabal block and 1(Ff19) from Qaimoh block.

Key words: Bakanae, *Fusarium fujikuroi*, pathogenic, variability, *Oryza sativa*

Introduction

Bakanae or foot rot disease caused *Fusarium fujikuroi* Nirenberg [teleomorph: *Gibberella fujikuroi* (Sawada) Ito] is one of the important and widely distributed diseases of rice which considerably limit its yield potential (1-4). In India the disease is emerging as one of the major biotic stresses of rice and has been reported to cause moderate to severe yield losses ranging from 15-25 per cent from different states of India (5-7). The disease for the last few years has been noticed regularly affecting rice crop in Kashmir, inflicting heavy economic losses particularly on *Japonica* type of cultivars cultivated under high altitude conditions of Kashmir valley (8). The introduction of this disease in Kashmir through seed material is quite possible owing to its seed borne nature. The disease is reported to be both soil (9, 10) as well as seed-borne (11-13). Since, the disease is primarily reported to be seed-transmitted, seed dressing represents the first way to control the spread of the disease. However, seed treatment alone fails to prevent soil-borne infection after transplanting which results in poor management of the disease (14). Prevention of soil-borne infection after transplanting is always difficult due to prolonged saprophytic survival of the fungus and impracticality of chemical approach to eradicate soil borne inoculum. Morever, long term

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usage of fungicides has led to the resistance development in pathogens (15, 16) besides environmental consequences associated with their use (17). The most efficient and economical method to mitigate the menace of bakanae disease is therefore, the use of resistant varieties. Since, bakanae disease in Kashmir has been recently found affecting the rice crop, the breeding programme has never been targeted at bakanae disease resistance. The success of developing varieties with durable disease resistance largely depends upon identification of genotypes with resistance against range of virulence present in pathogen population prevalent in target locations. The study was therefore conducted to determine the extent of pathogenic variability present in pathogen population under Kashmir condition which shall guide plant breeders to develop varieties with durable resistance to bakanae disease.

Materials and Methods

Status of Bakanae Disease: Survey of the rice growing areas of two districts of Kashmir valley viz., Anantnag and Kulgam was carried out during July to August, 2014 to assess the status of the bakanae disease. Three representative villages were taken from each of the three blocks of the district and three random paddy fields selected from each village. Hundred random plants from each field were selected for recording observations on incidence of the disease. The per cent disease incidence was calculated by using the following formula:

$$\text{Per cent disease Incidence} = \frac{\text{No. of plants infected}}{\text{No. of plants examined}} \times 100$$

***Collection, Isolation and Maintenance of Pathogen Isolates*:** Bakanae affected rice plants representing 8 rice genotypes and 17 diverse locations were collected during the course of survey and attempted for isolation of the causal pathogen. Twenty monoconidial cultures of *F. fujikuroi* (Table-1) were obtained using single spore isolation technique and maintained on Potato Dextrose Agar medium (PDA) for further studies. The pathogenicity test of the all the isolates of *F. fujikuroi* was established by confirming Koch' postulates on potted rice seedlings using their respective genotype from which they were isolated.

***Pathogenic Variability*:** Virulence spectrum of twenty isolates of *F. fujikuroi* was characterized using seed dip inoculation technique on a set of seven putative differential rice lines *viz*., K-332, Pusa Basmati-1509, GS-88, SK-407, SK-423, Mushkbudgi and GSL-225. The differential rice lines were selected on the basis of consistency in their reaction type against the test isolates. Seeds of each differential line, collected from apparently healthy plots were sterilized and inoculated separately with twenty test isolates by soaking the germinated seeds in the concentrated spore suspension of *F. fujikuroi* for 12 hours. Twenty inoculated seeds of each differential line were then sown in plastic trays, filled with sterilized soil in such a way that the differential set inoculated with same isolate accommodated in one tray. Uninoculated checks in which seeds were treated with sterilized distilled water were also maintained for each test isolate. The trays were incubated in plant growth chamber at 30±2°C day and 25±2°C night temperature, 80±5 per cent relative humidity and alternate periods of 12 hour light and 12 hour darkness and kept under observation for a period of 30 days. The observation on per cent incidence of bakanae infected plants was recorded after 30 days of sowing to assess the level of resistance and susceptibility of each test entry according to disease rating scale as adopted by (18), where, lines up to 40 per cent disease incidence were treated as resistant and with more than 40 per cent disease incidence as susceptible.

Results and Discussion

Disease Status: Bakanae disease was found prevalent in all the surveyed rice growing locations (Table-2). Occurrence of this disease of rice has been reported from all over the world

Table 1. Sources of *Fusarium fujikuroi* isolates

Isolate	Location	District	Block	Rice Genotype
Ff1	MCRS*, SKUAST-Kashmir, Larnoo	Anatnag	Larnoo	GSL-64
Ff2	MCRS*, SKUAST-Kashmir, Larnoo	Anatnag	Larnoo	GSL-19
Ff3	Khreti	Anantnag	Larnoo	K-332
Ff4	Larnoo	Anantnag	Larnoo	K-332
Ff5	Drawa	Anantnag	Larnoo	K-332
Ff6	Nagam	Kulgam	DH Pora	K-332
Ff7	Khul Ahamdabad	Kulgam	DH Pora	K-332
Ff8	Mandgur	Kulgam	DH Pora	K-332
Ff9	Badoora	Anantnag	Achabal	China-1039
Ff10	Khudwani	Kulgam	Qaimoh	China-1039
Ff11	Thajwara	Anantnag	Achabal	China-1039
Ff12	Qaimoh	Kulgam	Qaimoh	Jehlum
Ff13	Kreeri	Anantnag	Larkipora	Jehlum
Ff14	Chatarpora	Kulgam	Pahloo	Jehlum
Ff15	Nowpora	Kulgam	Larkipora	Jehlum
Ff16	Gasren	Kulgam	Pahloo	K-39
Ff17	Bugam	Kulgam	Qaimoh	K-39
Ff18	Achabal	Anantnag	Achabla	K-78
Ff19	MRCFC** (SKUAST-K), Khudwani	Kulgam	Qaimoh	Pusa Basmati-1509
Ff20	Zangalpora	Anantanag	Pahloo	Local

*MCRS: Mountain Crop Research Station
**MRCFC: Mountain Research Centre of Field Crops

(1, 19) including India (20, 21, 22, 8). The disease incidence however, varied from location to location which ranged from 0.6 per cent to 19.3 per cent. While studying the incidence of bakanae disease (21) also recorded varied levels of disease incidence ranging from 3.8 to 13.6 per cent in Rajasthan. Similarly, (23) recorded 0.5 to 12.5 per cent bakanae disease incidence from Peninsular Malaysia and three provinces of Indonesia. The varied levels of disease incidence observed at different surveyed locations may be attributed to different cultural and management practices adopted by the farmers and above all the type of cultivar being cultivated by the farmers which vary in their level of susceptibility and resistance. Effect of different cultural practices on bakanae disease incidence has been reported earlier (24, 25). Bakane disease management in rice crop through delayed sowing has been reported by (25, 26), while as (24, 27) found higher levels of nitrogen and potassium suppressing the bakanae pathogen. The average disease incidence was higher (8.7%) in district Anantnag than district

Table 2. Incidence of bakanae disease of rice at different locations of Anantnag and Kulgam districts of Kashmir during 2014.

District	Block	Location	Varieties grown	Bakanae Incidence(%)*
Anantnag	**Achabal**	Achabal	China-1039, K-78	7.3
		Thajwara	China-1039, K-78	6.0
		Badoora	China-1039, Jehlum	5.3
		Mean		**6.2**
	Larkipora	Doru	Jehlum, K-78	0.6
		Kreeri	China-1039, Jehlum	4.3
		Nowpora	China-1039, Jehlum	6.6
		Mean		**3.8**
	Larnoo	Larnoo	K-332	19.3
		Drawa	K-332	12.6
		Khreti	K-332	17.0
		Mean		**16.3**
Overall mean				**8.7**
Kulgam	**DH Pora**	KhulAhamabad	K-332	5.3
		Nagam	K-332	4.6
		Mandgur	K-332	4.6
		Mean		**4.8**
	Qaimoh	Khudwani	China-1039, Jehlum, Local	8.3
		Qaimoh	China-1039, Jehlum	4.3
		Bugam	K-39, Jehlum	7.3
		Mean		**6.6**
	Pahloo	Chatarpora	China-1039, Jehlum	3.3
		Gasren	China-1039, Jehlum, K-39	5.0
		Zangalpora	China-1039, Local	5.6
		Mean		**4.6**
Overall mean				**5.3**

*Average of three sites

Kulgam (5.3%). Maximum disease incidence was recorded at Larnoo (19.3 %) location followed by Khreti (17.0%) and Drawa (12.5 %) areas of district Anantnag, while as the lowest disease incidence was recorded at Doru (0.6%) location of district Anantnag. The highest disease incidence observed at Larnoo may be attributed to the cultivation of susceptible variety K-332 and use of infected and untreated farmers own saved seed. However, the low disease incidence (4.8 %) in the same type of cultivar, K-332 at DH Pora of district Kulgam and lowest disease incidence at Doru location was attributed to use of treated seeds as revealed by the farmers. Effectiveness of fungicide seed treatment in disease management has been reported by various workers (8, 16).

***Pathogenic Variability*:** Pathogenic variability study of 20 *F. fujikuroi* isolates collected from diverse locations was conducted on a set of

Table 3. Pathogenic grouping of *Fusarium fujikuroi* isolates using putative differential rice lines

Isolates	Reaction response of differential rice lines							Pathogenic Group
	K-332	Pusa Basmati -1509	GS-88	SK-407	SK-423	Mushk budgi	GSL-225	
Ff13, Ff15	S	R	R	S	R	R	R	I
Ff1, Ff2, Ff3, Ff4, Ff5	S	S	R	S	R	R	R	II
Ff9, Ff11, Ff16	S	S	R	S	S	R	R	III
Ff14, Ff18, Ff19, Ff20	S	S	R	S	S	R	S	IV
Ff10, Ff12, Ff17	S	S	R	R	S	S	R	V
Ff6, Ff7, Ff8	S	S	R	R	S	S	S	VI

seven putative rice differential lines. The twenty test isolates discern into six pathogenic groups on the basis of 100 per cent similarity in the reaction pattern exhibited by rice differential lines against these test isolates (Table-3). Pathogenic variability in bakanae pathogen has been reported earlier by various workers (1, 27-31). Amatulli et al. (31) found considerable pathogenic variability among the 28 isolates of *F. moniliforme* using five paddy cultivars as differential host. Pathogenic variability in *F. fujikuroi* isolates in terms of variable symptom expression has been also observed (29, 31, 32). The pathogenic groups more or less related to their respective geographical regions of collection. Two isolates from Block Larkipora (Ff13 and Ff15) shared a single group *i.e,* Group I and five isolates (Ff1, Ff2, Ff3, Ff4 and Ff5) from Larnoo block accommodated in Group II, Similarly, three isolates (Ff10, Ff12 and Ff17) from Block Qaimoh and three isolates (Ff6, Ff7 and Ff8) from block D.H.Pora shared group V and VI, respectively. The region specificity of isolates in terms of their virulence can be attributed to non dispersal of infected seed through farmer to farmer exchange of seed material and very narrow cultivar diversity over large cultivated area. The similarity of the isolates representing the particular geographical region has been also found by (33). Group III accommodated three isolates, two are from Achabal block (Ff9 and Ff11) and one from Pahloo block (Ff16) and Group IV accommodated 4 isolates, two (Ff14 and Ff20) from Pahloo block, one (Ff18) from Achabal block and one (Ff19) from Qaimoh block. The sharing of common pathogenic group by isolates from different blocks may be ascribed to farmer to farmer infected seed sharing from one block to another and to the broad genetic diversity of cultivated rice varieties in these areas. The variability within the isolates of same district has also been reported (34).

Conclusion

Bakanae disease caused *Fusarium fujikuroi* is one of the important, newly identified and widely distributed diseases of rice which considerably limit its yield potential. The survey was conducted to assess the status and pathogenic variability of the bakanae disease in the southern Districts of Kashmir valley. The disease incidence was found prevalent in all the rice locations which were surveyed. Pathogenic variability study of *F. fujikuroi* isolates collected from diverse locations was conducted on a set of seven putative rice differential lines.

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Ecofriendly Synthesis of Silver Nanoparticles by using Indian Plants and Screening their Catalytic Activity

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Abstract

Biosynthesis of silver nanoparticles using plants and their application in the field of medicine and agriculture has gained compliance in this decade. According to earlier research records the plant mediated silver nanoparticles (AgNPs) have also shown dye degradation and catalytic property in addition to antimicrobial, anticancer and antipest activities. The present study aims at synthesis of AgNPs using the aqueous leaf extracts of *Cascabela thevetia* and *Wrightia tomentosa* as well as whole plant aqueous extract of *Stemona tuberosa* Lour in individual reactions. The synthesized AgNPs were turned in to deep reddish brown after 48hrs incubation. Synthesis of AgNPs of all the three plants were conformed in UV analysis with the absorbance recorded at 436.09nm, 440.05nm and 435.04nm for *C. thevetia*, *W. tomentosa* and *S. tuberosa* Lour. Characterization of the AgNPs was carried out by Transmission electron microscope and Dynamic light scattering particle size analyser. Transmission electron microscopic images revealed that the AgNPs of all the plants were almost spherical in shape with varied sizes. In addition the AgNPs of three plants were screened for catalytic dye reduction activity and removal using 0.1mM methylene blue. The AgNPs of all three plants showed catalytic dye reduction activity and but AgNPs of *S. tuberosa* Lour have exhibited greater activity than to AgNPs of *C. thevetia* and *W. tomentosa.*

Key words: AgNPs, Dynamic light scattering, Catalytic activity, Methylene blue, *Stemona tuberosa* Lour

Introduction

Nanobiotechnology is an interdisciplinary branch of nanotechnology and biology that deals with the experimental studies for the synthesis of metal nanoparticles and their application in the field of medicine, agriculture, cleaning the environment (1). Biosynthesis of metallic nanoparticles has attracted attention due to their remarkable physiochemical and biological properties (2). In general Silver (Ag), Gold (Au), Zinc (Zn) and Platinum (Pt) were often used among metals in the biological synthesis of nanoparticles. It is very easy to synthesize metal nanoparticles of defined size and morphology by physical and chemical methods. However the biosynthesis of metallic nanoparticles is an alternative to physical and chemical methods because of its cost effective and ecofriendly nature (3). The plant mediated synthesis of metal nanoparticles is popular to other biological methods that require complicated procedures like culturing and maintenance of cells (4). Green (plant mediated) synthesis the metal nanoparticles involve the mixing of aqueous plant extract with the desired metal salt solution (5). Among all metals used in the synthesis of nanoparticles, Silver is the metal of choice in the biological systems due to its non toxicity to humans. In addition silver nanoparticles exhibited

remarkable antibacterial, antifungal, anticancer and antipest activities (6).

Cascabela thevetia is an evergreen tropical shrub belongs to the family Apocyanaceae. It is commonly called yellow oleander and cultivated throughout the world as ornamental plant. The plant contains glossy green and linear-lanceolate leaves (7). Nextone *Wrightia tomentosa* (Roxb.) Roem and Schult is an endangered medicinal tree and belong to the family Apocyanaceae. The plant grows in India and the plant parts are used in treatment of stomach ache, toothache, fever, haemorrhage and snake bite (8). *Stemona tuberosa* Lour is an herbaceous plant and belongs to the family Stemonaceae. The plant is seen in Central China, Indochina, Taiwan and India. The tuberous roots of the plant contain alkaloids and used to treat bacterial and helminthic diseases (9).

Synthetic dyes are used in most of the textile and paint industries as well as research laboratories (10). Huge quantities of the synthetic dyes that are used in these industries are not suitable for further use and are released in to the environment which is an issue of severe concern (11). These serious problems in the environmental ecosystem should be addressed and methods for degradation and removal of these dyes should be applied to clean the environment. Hence new treatment methods are required for the eradication and degradation of the persistent pollutants and conversion in to harmless products (12). The present study is undertaken for the synthesis of AgNPs from plant extracts of *Cascabela thevetia* and *Wrightia tomentosa* as well as whole plant aqueous extract of *Stemona tuberosa* Lour in individual reactions applying the green methods. Methylene blue is a thiazine dye and its cationic form is used as anti malarial agent and chemotherapeutic agent in the aquaculture industry. In addition it is also used in the analysis of trace levels of sulphide ions in aquatic samples, surgery, microbiology and diagnostic field (13, 14, 15). The synthesized plant mediated AgNPs have been evaluated for the photocatalytic degradation of methylene blue.

Materials and Methods

***Chemicals, reagents and plant source*:** The leaves of *Cascabela thevetia* were collected from Acharya Nagarjuna University campus, Guntur, India and leaves of *Wrightia tomentosa* and *Stemona tuberosa* Lour whole plant was collected from Tirumala hills, Tirupathi, India. The plants were taxonomically identified and authenticated by Prof. M. Vijayalakshmi, Dean and Professor, Dept of Botany, Acharya Nagarjuna University, Guntur, Andhra Pradesh, India. Silver nitrate ($AgNO_3$) with 99.5% purity and Methylene blue was purchased from Merck. Molecular grade water (Millipore, Milli Q) was used throughout the experimental studies. All the glassware used in the present study was carefully acid washed and rinsed with Milli Q water.

***Preparation of the aqueous plant extracts*:** The leaves of *C. thevetia* and *W. tomentosa* as well as whole plant of *S. tuberosa* Lour were washed thrice with distilled water, shade dried on a blotting paper, the dried leaves were finely chopped into small pieces and coarsely powdered with suitable pulveriser. 3gms of leaf powder *C. thevetia* is mixed with 100ml of distilled water and boiled at 100^0C for 10 minutes and the extract is filtered with Whatman NO: 1 filter paper. The same procedure is fallowed to prepare the aqueous extract of *W. tomentosa* and *S. tuberosa* Lour also. The clear pale yellow plant extract was used for further AgNPs production.

***Biosynthesis of AgNPs from the extracts of C. thevetia, W. tomentosa and S. tuberosa* Lour:** AgNPs of all three plants described above were synthesized in individual experiments by adding 10ml of aqueous plant extract to 190ml of readily prepared 1mM silver nitrate solution ($AgNO_3$). The suspension is stirred using a magnetic stirrer for 20 min and kept for incubation for 48h at room temperature. The change in the colour of the solution was observed from light yellow to dark brown indicating the termination of the reaction.

***Characterisation of AgNPs*:** The formation of silver nanoparticles was confirmed by UV-Visible

spectroscopic studies after 48hrs using $AgNO_3$ solution as blank. Spectral analyses of AgNPs synthesized from all three plants were studied using UV-VIS Double beam spectrophotometer (Thermo Fischer) and the values were recorded in the range of 300 to 600 nm. To know the morphology and particle size distribution the synthesized AgNPs were centrifuged at 15000 rpm for 10 minutes, dried and the fine powder was placed in a carbon coated copper grid and analysed in a Transmission electron microscope (TEM). Dynamic light scattering measurements of the synthesized AgNPs after 120hrs incubation were carried out using HORIBA SZ-100 particle size analyzer to estimate the average size distribution of the prepared particles.

***Catalytic Activity*:** The catalytic activity of AgNPs synthesized from all three plants was studied in individual experiments using milli Q water as blank and the absorbance values were recorded using UV-Visible spectrophotometer (Thermo scientific). 1ml of 0.1mM methylene blue was mixed with 2ml of milli Q water and the absorbance maximum was recorded. In the first reaction 1ml of 0.1mM methylene blue was mixed with 0.2ml of plant extract and 1.8ml of milli Q water and kept for incubation. In the second reaction 1ml of methylene blue of same concentration was mixed with 0.2ml of plant extract and 1.8ml of synthesized AgNPs and kept for incubation. In all the reactions the total volume was made up to 3ml. The absorbance maxima of incubated first and second reactions were recorded after 30, 40 and 50 minute intervals. The values obtained were compared with the absorption maximum of pure methylene blue (16).

Results and Discussion

For synthesis of silver nanoparticles,10 ml of aqueous plant extract is added to 190 ml of $AgNO_3$ solution and incubated for 48 hours. After incubation, the suspension turned in to deep reddish brown in colour which is an indication of the completion of reduction reaction resulting in the formation of AgNPs of *C.thevetia, W.tomentosa* and *S. tuberosa* Lour (Fig. 1) (17).

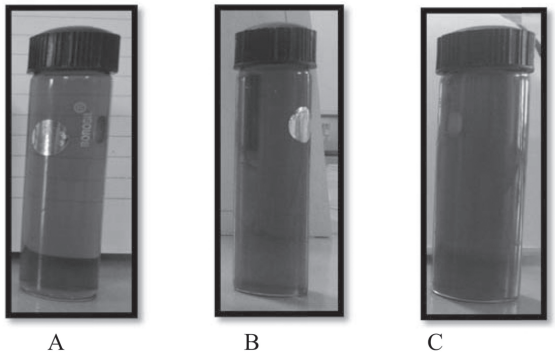

Fig. 1. A) AgNPs of *Cascabela thevetia*
B) AgNPs of *Wrightia tomentosa*
C) AgNPs of *Stemona tuberosa* Lour

The plant extract acts as a reducing, stabilising and capping agent for the synthesis of silver nanoparticles (18).

***UV-Vis Analysis*:** UV-Visible analysis the AgNPs of *C. thevetia*, *W. tomentosa* and *S. tuberosa* Lour have shown the absorption maxima of 436.09nm, 440.05nm and 435.04nm due to surface Plasmon resonance (Fig:2). The obtained results are in agreement with the UV-Visible study results depicted and discussed to the green synthesized AgNPs of different plants (19, 20).

***TEM Analysis*:** TEM images of the leaf mediated AgNPs of the plants were shown in the Fig: 3. From TEM images it is shown that the AgNPs of *C. thevetia, W. tomentosa* and *S. tuberosa* Lour were not aggregated and mono dispersed (21), almost spherical in shape and size ranges 20-30nm, 60-70nm and 10-40nm respectively.

***DLS Studies*:** Dynamic light scattering (DLS) studies were conducted to investigate the hydrodynamic size of the plant mediated AgNPs in colloidal aqueous environment. DLS measurements of *C. thevetia*, *W. tomentosa* and *S. tuberosa* Lour AgNPs were taken after 120hrs incubation (Fig 4). The particles have shown brownian motion when dissolved in the medium

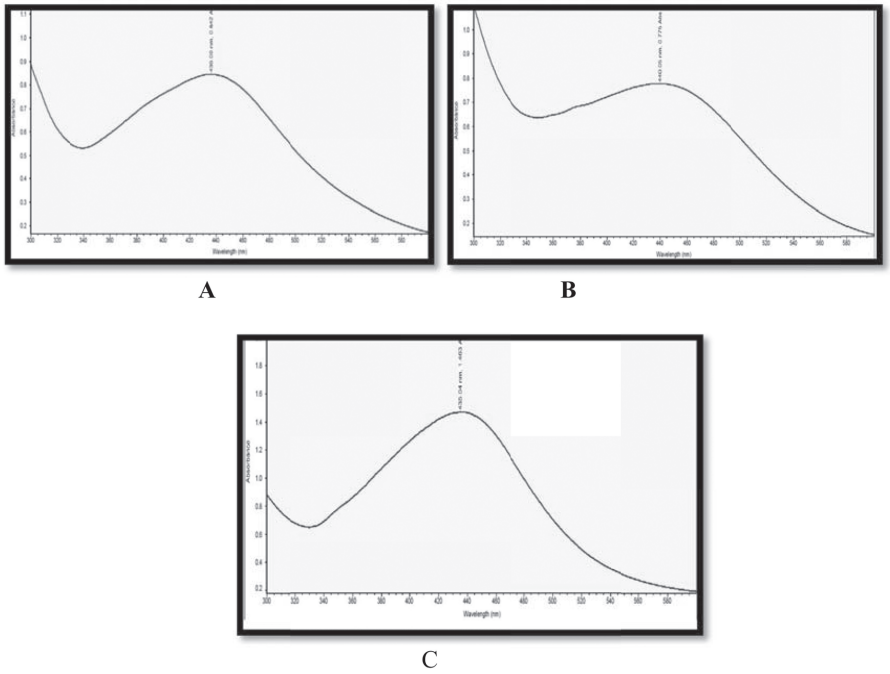

Fig. 2. (A), (B) and (C) represents UV-Visible spectrum at 436.09nm, 440.05nm and 435.04nm of *C. thevetia*, *W. tomentosa* and *S. tuberosa* Lour AgNPs

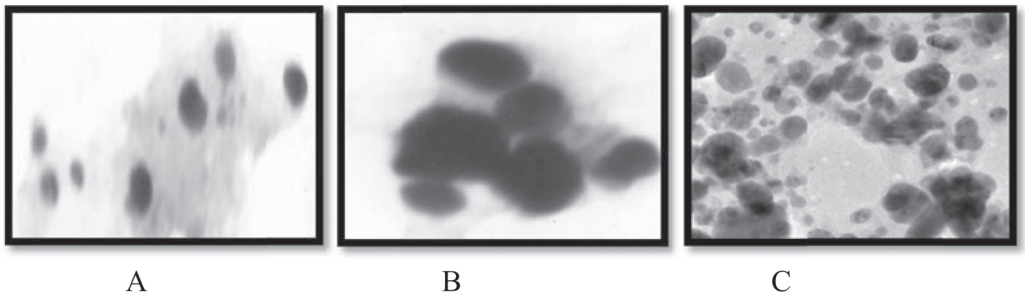

Fig. 3. A, B and C represents TEM images of *C. thevetia*, *W. tomentosa* and *S. tuberosa* Lour AgNPs respectively

which is measured by the variations in the intensity of scattered light in the system from which translational diffusion co-efficient is calculated by applying Stokes-Einstein equation which determines the hydrodynamic size (22). The study was carried out at 25°C in a standard monodispersed medium maintained at a viscosity of 0.892 m Pa·s. The histograms (HORIBA SZ-100 particle size analyser) of the AgNPs of *C. thevetia*, *W. tomentosa* and *S. tuberosa* Lour obtained indicates that the average size of nanoparticles was 1.7nm, 41.5nm and 0.4nm respectively (23). A PDI value more than 0.5 refers to the aggregation of the particles. Polydispersity index represents the ratio between different sizes to total number of particles (24).

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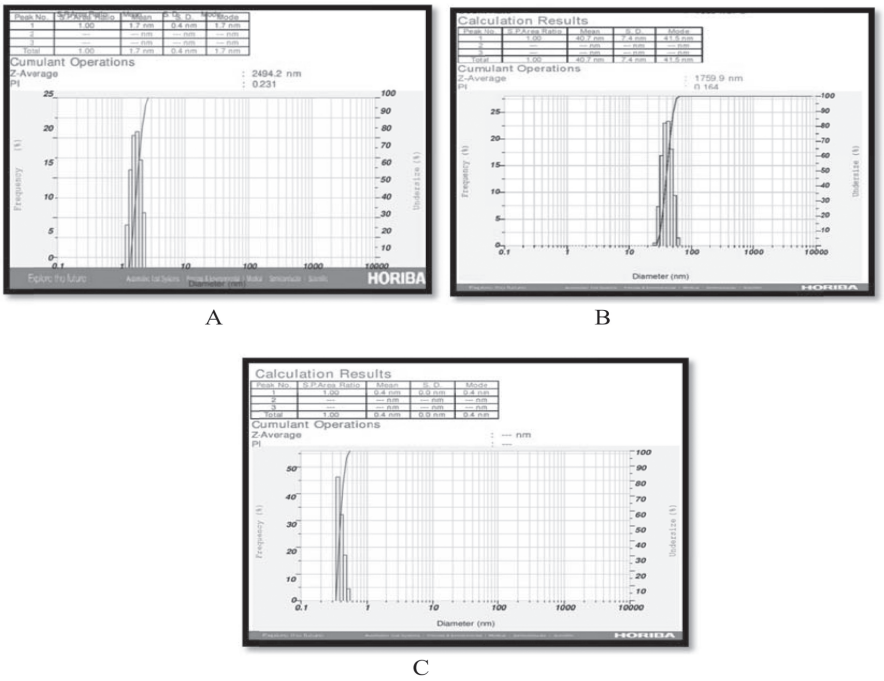

Fig. 4. A) DLS of *C. thevetia* AgNPs B) DLS of *W. tomentosa* AgNPs C) DLS of *S. tuberosa* Lour AgNPs

The PDI values of *C. thevetia*, *W. tomentosa* and *S. tuberosa* Lour AgNPs is less than 0.3 and indicates that the nanoparticles are monodispersed in colloidal aqueous solution.

***Catalytic Activity*:** Many reports suggest the plant mediated AgNPs has significant catalytic activity for dye reduction and removal (25, 26, 27). The plant mediated AgNPs of *C. thevetia*, *W. tomentosa* and *S. tuberosa* Lour were studied for methylene blue dye reduction. Pure methylene blue of 0.1mM concentration showed a lambda max value at 664nm. When first reaction of *C. thevetia*, *W. tomentosa* and *S. tuberosa* Lour was analyzed in UV-Visible spectrophotometer the absorbance is gradually decreased and shifted to higher wavelength compared to pure methylene blue. It indicates that the plant extract of all three plants have ability to degrade methylene blue. The second reaction of *C. thevetia*, *W. tomentosa* and *S. tuberosa* Lour was also analyzed for lambda max value after 30 minutes. The absorption further decreased and shifted to higher wavelengths when compared to first reaction. The second reaction of both plants again screened for absorption maxima after 40minutes as well as 50 minutes incubation. The absorption is gradually decreased and shifted to higher wavelength after 40 and 50 minutes incubation. The results were depicted in the figure 5 and the graphs obtained clearly indicate that AgNPs of *C. thevetia*, *W. tomentosa* and *S. tuberosa* Lour have shown catalytic activity in degradation and removal of methylene blue. Comparative analysis of AgNPs of *S. tuberosa* Lour exhibited greater catalytic activity where as the AgNPs of *W. tomentosa* and *C. thevetia* exhibited moderate and poor catalytic activity respectively in degradation and removal of methylene blue.

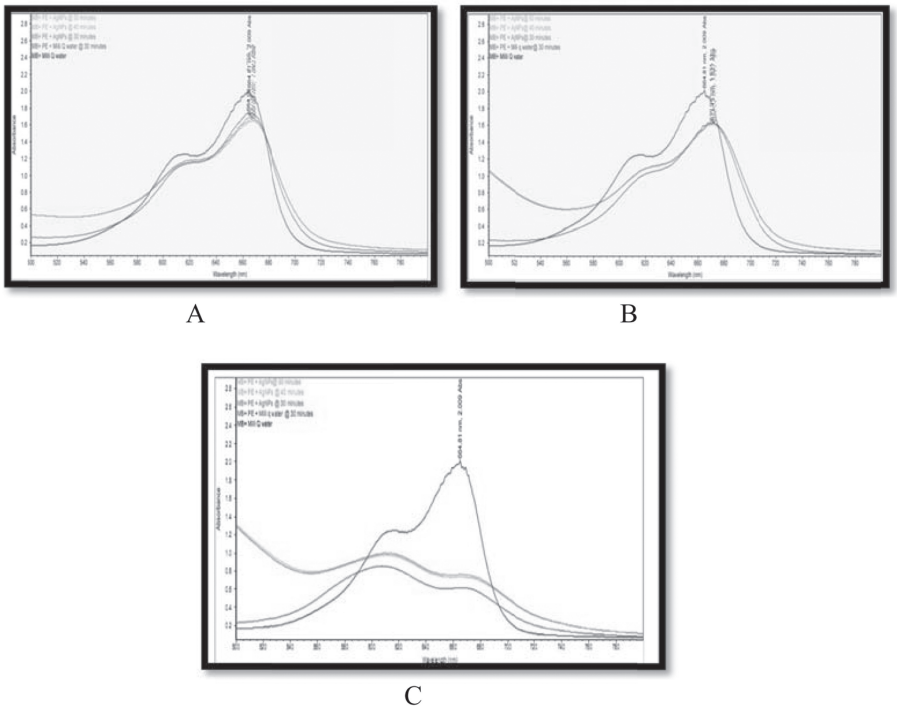

Fig. 5. A, B and C represents Catalytic activity of *Cascabela thevetia*, *Wrightia tomentosa* and *S. tuberosa* Lour AgNPs on methylene blue

Conclusion

Biosynthesis of silver nanoparticles using plants is a cost effective, time saving and ecofriendly approach than to physical and chemical methods and other biological methods used in the synthesis of silver nanoparticles. In the present study AgNPs were synthesized using the leaves of *Cascabela thevetia*, *Wrightia tomentosa* and *Stemona tuberosa* Lour in individual reactions. The formation of AgNPs from all plant extracts was confirmed in UV-Visible spectrophotometer with the absorption peaks at 436.09nm, 440.05nm and 435.04nm respectively. Dynamic light scattering analysis indicates that the average size of *C. thevetia* AgNPs was 1.7nm, *W. tomentosa* AgNPs was 41.5nm and *S. tuberosa* Lour was 0.4nm. The catalytic activity of leaf mediated AgNPs of all plants is shown by the methylene blue dye degradation and removal. The AgNPs of *S. tuberosa* Lour have shown greater catalytic activity with methylene blue than the AgNPs of *C. thevetia*, *W. tomentosa*.

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Nutritional Evaluation and Mineral Elements Analysis of Threatened Medicinal Plants *Boucerosia indica* (Wight & Arn.) Plowes and *Caralluma adscendens* (Roxb.) R.Br. var. *fimbriata* Gravely & Mayur.

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Abstract

Plants are the rich source of all the nutrients, minerals and elements essential for human beings. Explorations of less known edible plants should be carried out and their cultivation, using modern agricultural practices, may supplement our requirement of food. Technical studies on their nutritional values are needed before they can be suggested as sources of non conventional foods to boost food supply. *Boucerosia* and *Caralluma* species are xerophytic succulents belonging to the Apocynaceae family with approximately 100 species distributed worldwide. These plants are found in drier regions of India and used as a part of famine food and for antiobeity. *Boucerosia indica* and *Caralluma adscendens* var. *fimbriata,* being succulent, showed high percentage of moisture content, minimizing the amount of total solids to a meager. Ash content was found to be moderate. By the present research we found considerable concentrations of various major and minor mineral elements such as: Sodium, Potassium, Calcium, Magnesium, Phosphorous, Zinc, Manganese, Iron, but low levels of Chromium and Lead respectively. The above two plants contained low levels of Fat, moderate amounts of Protein and Fibre, rich source of Carbohydrate. Finally the Nutritive value is very high in both the plants.

Key words: *Boucerosia indica* and *Caralluma adscendens var. fimbriata,* nutritional value, mineral elements

Introduction

Plants have been used as the source of food from the time when human existence started on earth. Since time immemorial, traditional knowledge and indigenous evidences suggest that a variety of wild edible plant species in India have played a prominent role in providing food and medicine for human beings as well as animals. Edible plants may have different uses in different areas of the same country. Cultivated plants are widely used today although wild edible plants have the significant medical and the other economical properties. During the recent past, wild edibles have featured outstanding in the discussions and framework of rural development and biodiversity conservation. Poor rural and tribal people depend on a wide variety of plants, animals and fungi for their own consumption and for income generation. The South Indian region is well known for these plants which are being utilized by local inhabitants for various purposes, i.e., medicine, fuel, food, timber etc. The nutritive value of plant plays great role in plant and human being, so material extracted from the natural plant through chemical or biotechnology method was elucidated by Chapman (1).

All human beings require a number of complex organic compounds as added calorific requirements to meet the need for their all muscular activities. Humans require a suite of mineral elements in varying amounts for proper growth, health maintenance and general well-being. Human body comprises chemical

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compounds such as water, proteins, fatty acids, nucleic acids and carbohydrates; these in turn consist of elements such as carbon, hydrogen, oxygen, nitrogen and phosphorus etc. and minerals. Majority of the wild edible plants having carbohydrates, fats, fibre and proteins form the major portion of the diet, while minerals and vitamins form comparatively a smaller part (2). Plants are capable of absorbing a wide range of mineral ions with relevance to human nutrition and health. Well balanced diet that includes mixed sources of grains, fruits and vegetables, plant foods can make a significant contribution to daily nutritional and mineral needs at all stages of the life cycle. Unfortunately, consumption patterns are not always ideal, and many individuals both in developed and developing countries are failing to attain recommended nutritional and mineral intakes.

There are some important reports regarding mineral and nutritional analysis of plants. Jain *et al.* (3) have studied the analysis for mineral elements of some medicinal plants. Duhan *et al.* (4) extensively reported the value of some non conventional plant foods of India. Gopalan *et al.* (5) worked out on nutritive value of Indian foods. Indrayan *et al.* (6) worked on determination of nutritive value and analysis of mineral elements for some medicinally valued plants like, *Artocarpus heterophyllus, Nelumbo nucifera* etc. Nile and Khobragade (7) studied nutritive value and mineral elements of important medicinal plants like *Tinospora cordifolia*, *Gymnema sylvestre* etc. Umar *et al*. (8) studied nutritional composition of water spinach (*Ipomoea aquatica*) leaves. Sridhar and Bhat (9) have revealed about Lotus a potential nutraceutical source. Aberoumand and Deokule (10) have also focused on nutritional values of some wild edible plants like *Asparagus officinalis, Alocacia indica etc.*

Boucerosia and *Caralluma* species are growing in India, Africa, Middle East, Spain and Pakistan (11). Several species of these genera are found in India. Plants of these species are edible and the medicinal properties include; anti-inflammatory, anti-nociceptive, anti-ulcer, anti-diabetic, anti-pyretic and anti-oxidant effects. *Boucerosia* and *Caralluma* extracts have also been found to be appetite suppressant, a property which is well known to Indian tribals and some *Caralluma* species are used in the treatment of obesity. The extracts of *Caralluma* species in the form of capsules have been released under different trade names like GENASLIM, SLIMALUMA etc. for body weight control or obesity (11).

Pharmacological review of *Caralluma* with special reference to appetite suppression and antiobesity was studied by Dutt *et al*. (12). Kunert *et al*. (13) researched on pregnane glycosides of *Caralluma adscendens* var. *fimbriata.* Quantitative determination of pregnanes from aerial parts of *Caralluma* species using HPLC-UV and identification by LC-ESI-TOF was carried by Avula *et al*. (14).

Obesity is a major global health problem and a risk factor for several chronic disorders such as diabetes, hyperlipidemia, hypertension and cardiovascular diseases. Kuriyan *et al.* (15) extensively researched on effect of *Caralluma fimbriata* extract on appetite, food intake and anthropometry in adults. The huge benefits of the plant *Caralluma* well catered in Caralluma (16). Antiobesogenic and antiatherosclerotic properties of *Caralluma fimbriata* extract found by Soundararajan *et al.* (17). Plants belonging to this genus are rich in esterified polyhydroxy pregnane glycosides. The genus is also characterized by the presence of flavone glycosides (18). *Caralluma umbellata* has in vitro antibacterial activity and is used in traditional medicine by Indian tribes (19). Some selected species of *Caralluma* and *Boucerosia* showed antiadipogenesis activity, cellular antioxidant activity and pregnane steroid on cell lines (20, 21). Evaluation of antiproliferative properties of selected species of *Caralluma* and *Boucerosia* on skin cancer cell lines was carried out by Vajha *et al.* (22).

Recent research has highlighted the need for screening of nutritional and mineral properties of plants as they contain all the necessary mineral and nutrients which play a vital role in daily human diet and activities. The present study is therefore attempted to investigate the mineral and nutrient composition of two threatened medicinal plant species *Boucerosia indica* and *Caralluma adscendens* var.*fimbriata* (Fig. 1 and 2). Many researchers have tried to determine the nutritive values and mineral composition of many medicinal plants, but to the best of our knowledge no reports are available on the mineral and nutritional studies of *Boucerosia* and *Caralluma*. Fortunately, research in plant mineral nutrition is ongoing globally and will continue to receive much attention, because efforts to improve plant mineral status are important not only for the nutritional value of our food supply, but also for the healthy and reproductive output of our agronomic crops. However, more advanced, effective pharmacological and clinical studies would be required to investigate *in vivo* mechanism of nutraceutical effects of these very important wild plant species.

Fig. 1. *Boucerosia indica*

Fig. 2. Natural habitat of *Caralluma adscendens var. fimbriata*

Materials and Methods

***Mineral and Trace Elements Analysis*:** Plant materials (Fig. 1 and 2) were collected, washed with lukewarm water and dried in shade. To prepare the sample for mineral analysis, the washed and dried materials were ground to fine powder and used for dried ashing. In each case the powdered plant material was taken in a pre cleaned and constantly weighed silica crucible and heated in a muffle furnace at 400°C till there was no evolution of smoke. The crucible was cooled at room temperature in a desiccator and carbon-free ash was moistened with concentrated sulphuric acid and heated on a heating mantle till fumes of sulphuric acid ceased to evolve. The crucible with sulphated ash was then heated in a muffle furnace at 600°C till the weight of the content was constant (~2–3 h). 1 g of sulphated ash obtained above was dissolved in 100 ml of 5% HCl to obtain the solution ready for determination of mineral elements through atomic absorption spectroscopy (AAS) and flame photometry (FPM). Standard solution of each element was prepared and calibration curves were drawn for each element using AAS/FPM.

***Proximate/ Nutritive Value Analysis*:** For determination of nutritive value, various parameters were studied using the crushed plant material through (AOAC) Association of the Official Analytical Chemists methods [23]. Moisture content, ash, crude fat, crude fiber, crude protein and carbohydrate contents and overall nutritive value contents was analyzed by standardized protocols (24).

***Determination of Moisture Content*:** For determination of moisture content, the sample

materials were taken in a flat-bottom dish and kept overnight in an air oven at 100–110°C and weighed. The loss in weight was regarded as a measure of moisture content.

***Determination of Ash Content*:** For determination of ash content, 10 g of each sample was weighed in a silica crucible. The crucible was heated first over a low flame till all the material was completely charred, followed by heating in a muffle furnace for about 3–5 h at 600°C. It was cooled in a desiccator and weighed to ensure completion of ashing. To ensure completion of ashing, it was heated again in the furnace for half an hour, cooled and weighed. This was repeated till the weight became constant (ash became white or greyish white). Weight of ash gave the ash content.

***Determination of Crude Fat*:** Crude fat was determined by extracting 2 g moisture free sample with petrol in a Soxhlet extractor, heating the flask on a sand-bath for about 6 h, till a drop taken from the drippings left no greasy stain on the filter paper. After boiling with petrol, the residual petrol was filtered using Whatman No. 40 filter paper and the filtrate was evaporated in a pre weighed beaker. Increase in weight of beaker gave crude fat.

***Determination of Crude Protein*:** The crude protein was determined using micro Kjeldahl method. 2 g of oven-dried material was taken in a Kjeldahl flask and 30 ml conc. H_2SO_4 was added followed by the addition of 10 g potassium sulphate and 1 g copper sulphate. The mixture was heated first gently and then strongly once the frothing had ceased. When the solution became colourless or clear, it was heated for another hour, allowed to cool, diluted with distilled water and transferred to an 800 ml Kjeldahl flask, washing the digestion flask. 3 or 4 pieces of granulated Zn and 100 ml of 40% caustic soda were added and the flask was connected with the splash heads of the distillation apparatus. Next 25 ml of 0.1 N sulphuric acid was taken in the receiving flask and distilled. When two-thirds of the liquid had been distilled, it was tested for completion of reaction. The flask was removed and titrated against 0.1 N caustic soda using methyl red indicator for determination of Kjeldahl nitrogen, which in turn gave the protein content.

***Determination of Crude Fibre*:** Crude fibre was determined to be reported along with the nutritive value. The estimation was based on treating the moisture and fat-free material with 1.25% dilute acid, then with 1.25% alkali. Then 2 g of moisture and fat-free material was treated with 200 ml of 1.25% H_2SO_4. After filtration and washing, the residue was treated with 1.25% NaOH. It was then filtered, washed with hot water and then 1% HNO_3 and again with hot water. The residue was ignited and the ash weighed. Loss in weight gave the weight of crude fibre.

***Determination of Carbohydrate*:** Percentage of carbohydrate was given by: 100 – (percentage of ash + percentage of moisture + percentage of fat + percentage of protein).

***Nutritive Value was Finally Determined by*:** Nutritive value = 4 x percentage of protein + 9 X percentage of fat + 4 X percentage of carbohydrate. The percentage of various mineral elements and nutritive values were summarized in tabular form.

Results and Discussion

The sample solution for mineral analysis was prepared first, then washed and dried materials were ground to fine powder and used for dried ashing. In each case the powdered plant material was taken in a pre cleaned and constantly weighed silica crucible and the same method was followed through, atomic absorption spectroscopy (AAS) and flame photometry (FPM). Results of the mineral and trace elements in two medicinal plants are given in Table- 1; Fig. 3. The concentrations of these elements for *Boucerosia indica* and *Caralluma adscendens* var*. fimbriata* are as follows; (w/w). Sodium (Na) is higher 65 mg/100 g in *B. indica* compared to *C. fimbriata* 40 mg/100 g. Sodium plays important role in transport of metabolites and takes part in ionic balance of human body.

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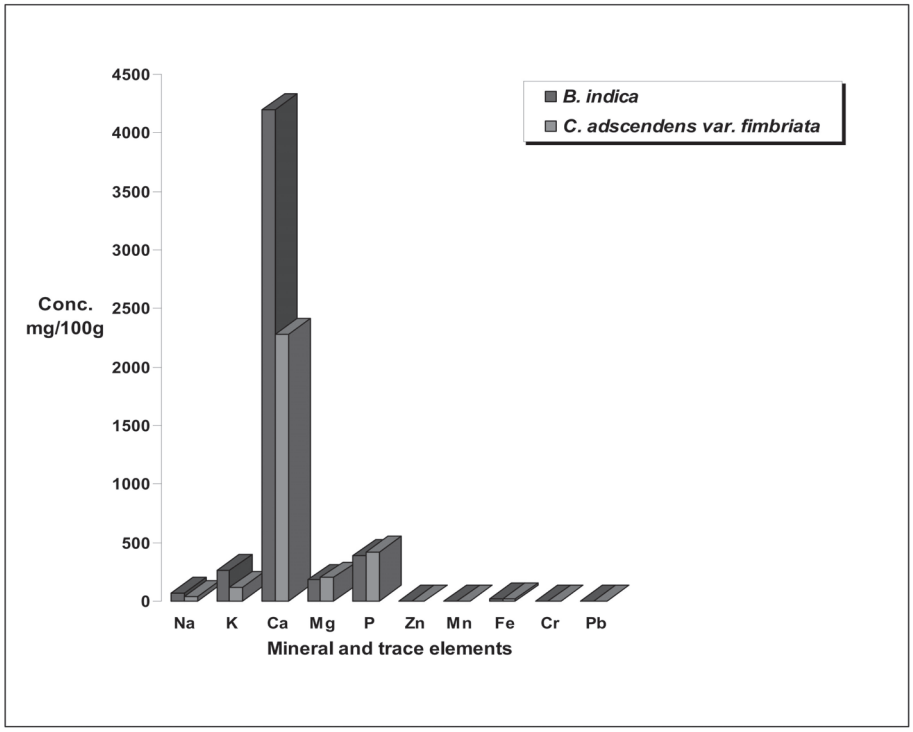

Fig. 3. Concentrations of various Mineral and trace elements

Potassium (K) is higher with 260 mg/100 g in *B. indica* than *C. fimbriata* with 120 mg/100 g. Potassium regulates heart rhythms, important as a diuretic, maintain tissue excitability and normal muscle contraction and helps in release of chemicals which acts as nerve impulses. Its deficiency causes nervous irritability mental disorientation, low blood sugar, insomnia and coma. Calcium (Ca) is also high in *B. indica* with 4200 mg/100 g and moderate in *C. fimbriata* 2280 mg/100 g. Calcium plays important role in building and maintaining strong bones, teeth and also large part of human blood and extra cellular fluids. It is also necessary for normal functioning of cardiac muscles, blood coagulation, milk clotting and regulation of cell permeability. Ca deficiency causes rickets, back pain, osteoporosis, indigestion, irritability, premenstrual tension and cramping of the uterus.

The concentration of Magnesium (Mg) is slightly low in *B. indica* with 180 mg/100 g than *C. fimbriata* with 200 mg/100 g. Magnesium is highly required in plasma and extracellular fluid, maintains osmotic equilibrium and plays important role in formation and function of bones, muscles, prevents disorders, high blood pressure and depression, also plays important role in enzyme activity. Mg deficiency interferes with transmission of nerve and muscle impulses, causing irritability and nervousness, prevent heart diseases. Phosphorous (P) is low in *B. indica* with 390 mg/100 g slightly high in *C. fimbriata* with 420 mg/100 g**.** Phosphorous maintains blood sugar level, normal heart contraction, cell growth and repair mainly needed for bone growth, acid base balance and kidney function.

Zinc (Zn) is quite low in *B. indica* with 0.31 mg/100 g compared to *C. fimbriata* which contains 0.40 mg/100 g. Zinc is a component of many metallo enzymes, including some enzymes which play a central role in nucleic acid metabolism. In addition, Zn is a membrane

stabilizer and a stimulator of the immune response. Its deficiency leads to impaired growth and malnutrition. Manganese (Mn) is also low in *B. indica* is 0.40 mg/100 g and high in *C. fimbriata* with 0.70 mg/100 g. Manganese is essential for hemoglobin formation, but excess is harmful. Iron (Fe) was comparatively low in *B. indica* with 20 mg/100 g, and in *C. fimbriata* it was 22 mg/100 g. Iron make body tendons and ligaments, certain chemicals of brain are controlled by presence or absence of Iron, it is essential for formation of hemoglobin, carries oxygen around the body. Fe deficiency causes anemia, weakness, depression, poor resistance to infection.

Chromium (Cr) was very low in comparison with all other mineral elements in both studied plants. *B. indica* contains 0.02 mg/100 g, whereas *C. fimbriata* contains 0.01 mg/100 g. Chromium is vital element as it works with insulin to stabilize blood sugar level, help to absorb energy from blood and increase muscle mass, reducing fat mass in human body, it plays a vital role in metabolism of carbohydrates. Deficiency of Cr results in growth failure, cataract, hyperglycemia, neuropathy, atherosclerosis and leads to diabetes in human. Lead (Pb) is also lowest among various biologically important elements. As it is 0.01 mg/100 g in *B. indica* and BDL (Below detectable level) in *C. adscendens* var. *fimbriata* Lead is best known for its toxicological properties. Low levels of Pb in both plants suggested their non toxic nature.

Nutritive values of *Boucerosia indica* and *Caralluma adscendens* var*. fimbriata* were high on a dry matter (DM) basis. The percentages of proximate analysis results (Table- 2; Fig. 4) revealed that dried part of *B. indica* has Moisture content 94.6 %, Ash content 22.52%, Crude Fat 3.54%, Crude Protein 11.58%, Crude fibre 15.00%, Carbohydrate 62.36% and finally Nutritive value 327.62 (cal/100gm). Where as in *C. adscendens* var. *fimbriata,* Moisture content was 95.10%, Ash content 15.81%, Crude Fat 3.90%, Crude Protein 10.82%, Crude fibre

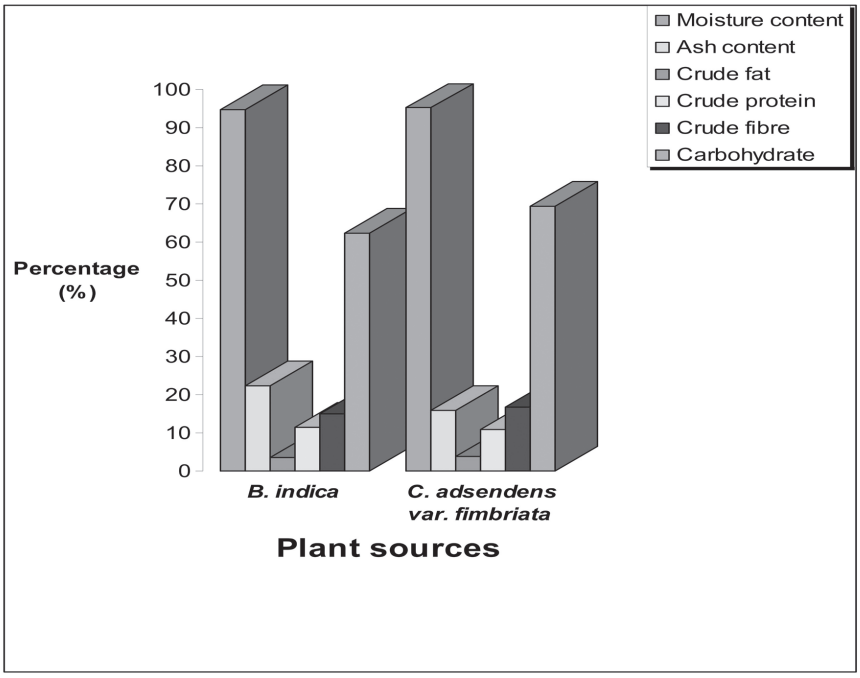

Fig. 4. Percentages of Proximate samples

Table 1. Mineral and trace elements composition of *Boucerosia indica* and *Caralluma adscendens* var. *fimbriata* by atomic absorption spectroscopy (AAS) and flame photometry (FPM)

Minerals mg/100 gm	***Boucerosia indica***	***Caralluma adscendens* var. *fimbriata***
Sodium (Na)	65	40
Potassium (K)	260	120
Calcium (Ca)	4200	2280
Magnesium (Mg)	180	200
Phosphorous (P)	390	420
Zinc (Zn)	0.31	0.40
Manganese (Mn)	0.40	0.70
Iron (Fe)	20	22
Chromium (Cr)	0.02	0.01
Lead (Pb)	0.01	BDL

Table 2. Proximate/Nutritive value composition of *Boucerosia indica* and *Caralluma adscendens* var. *fimbriata* by Association of the Official Analytical Chemists (AOAC) methods

Parameters	***Boucerosia indica***	***Caralluma adscendens* var. *fimbriata***
Moisture content (%)	94.61	95.10
Ash content (%)	22.52	15.81
Crude fat (%)	3.54	3.90
Crude protein (%)	11.58	10.82
Crude fibre (%)	15.00	16.82
Carbohydrate (%)	62.36	69.47
Nutritive value (cal/100gm)	327.62	356.26

16.82%, Carbohydrate 69.47% and lastly Nutritive value 356.26 (cal/100gm).

These two antiobesity plants contains considerable quantities of good source of essential nutrients, which supports their use as food, fodder and good source of various important nutrients for humans as well as live stock. *Boucerosia indica* and *Caralluma adscendens* var.*fimbriata* with high vegetable carbohydrate, fibre, sufficient protein and with low fat. And also with suitable mineral elements showing high nutritive value. The elevated moisture concentration of these two plants agrees with definitions of vegetables, which were characterized with high water content. The protein contains all of essential amino acids and forms the building blocks of bones, teeth, muscles, skin and blood. In addition, it helps to regulate fluid balance and acts as enzymes transporters. As an antibody, protein also helps as a defense mechanism of the body against different diseases. The body can synthesize many of the amino acids required for protein synthesis, but some amino acids must be obtained from the proteins in the diet. Plant food

that provides more than 12% of its calorific value from protein is considered as good source of protein. And mineral deficiency causes poor growth, impaired immune function and delayed mental development (25).

Adequate intake of dietary fibre (DF) plays an important role in decreasing the risks of many disorders such as constipation, diabetes, cardiovascular diseases, diverticulosis, obesity, serum cholesterol level, hypertension, colon and breast cancer (26). All the fractions (Cellulose, lignin, hemicellulose, pectin, gums and mucilage) of dietary fibre are the major constituents of plant cell wall. As such, carbohydrate is the most important source of food energy among the macronutrients, accounting between 40–80% of total energy intake (27). Fat is in tune with that a diet contain 1–2% of its calorific of energy as a fat is said to be sufficient to human beings while excess fat consumption is implicated to certain cardiovascular disorders (28). However, the present findings suggest that *B. indica* and *C. adscendens* var. *fimbriata* could have great potential in nutritive value together with its medicinal properties.

Summary and Conclusions

Plants are complex organisms whose mineral needs are determined by a number of molecular, cellular and whole-plant events. *Boucerosia indica* and *Caralluma adscendens* var*. fimbriata* species have many medicinal and pharmaceutical uses and grow abundantly in the Southern India, its sustainable harvest from nature and cultivation may improve the local economy. Moreover, the xerophytic nature of the species may have advantages in cultivating in dry slopes to protect the soil erosion. These present findings have important connotations in light of upcoming organic foods, nutraceutical and pharmaceutical industries in the state. Our research data show that both the plants contain appreciable amount of proteins, fat, fibre, carbohydrate and calorific value, mineral, trace elements and generally low levels of toxicants. Thus, it can therefore be concluded that *B. indica* and *C. adscendens* var. *fimbriata* can contribute significantly to the nutrient requirements of man and should be used as a source of nutrients to supplement other major sources. Chemical analysis however should not be the sole criterion for judging the nutritional value of these two plants. It is necessary to consider other aspects such as the biological evaluation of the nutrient contents of these plants in order to determine the bioavailability of the nutrients and also the effects of processing on the chemical and nutritive value of the plants.

***Mineral and Trace Elements Analysis*:** The concentrations of these elements for *Boucerosia indica* and *Caralluma adscendens* var*. fimbriata* (Table- 1; Fig. 3) are as follows: w/w (mg/100 g) Sodium - 65 and 40, Potassium 260 and 120, Calcium 4200 and 2280, Magnesium 180 and 200, Phosphorous 390 and 420, Zinc 0.31 and 0.4, Manganese 0.4 and 0.7, Iron 20 and 22, Chromium 0.02 and 0.01 and Lead 0.01 and BDL respectively.

***Proximate or Nutritive Value Analysis*:** *Boucerosia indica* and *Caralluma adscendens* var*. fimbriata* (Table- 2 & Fig. 4) contained 94.61% and 95.81% of Moisture, 3.54% and 3.90% Fat, 11.58% and 10.82% Protein, and 15.00% and 16.82% Fibre, 62.36% and 69.47% Carbohydrate respectively. Finally nutritive value was calculated as 327.62 Cal/100g and 356.26 Cal/100g for dry weight respectively.

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Stability Indicating Assay Method Development and Validation to Simultaneously Estimate Metformin Hydrochloride and Canagliflozin by RP-HPLC

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Abstract

The focus of this study is to develop and validate a HPLC method to simultaneously estimatemetformin hydrochloride and canagliflozin in bulk using GraceSmart RP-18 column (250× 4.6mm, 5µ)at 30°C.Combination of acetonitrile(ACN) and ammonium acetate bufferin the ratio of 45:55v/v with pH 4.5 was used as mobile phase with 1ml/min flow rate. It was detected by photo diode array detector at 252 nm. The retention timeobserved for metformin hydrochloride and canagliflozin were found to be 4.00 and 5.76 min respectively. The method was developed and found to be linear with correlation coefficients r^2 of 0.9993 and 0.9992 for metformin hydrochloride and canagliflozin respectively within a concentration range of 1-80µg/ml. Stability studies were performed by exposing the drugs to acidic, basic, oxidative, thermal and photolytic stress conditions with sampleswithdrawn at different time intervals. Analysis of the above samples were done by the developed method. The method to estimate metformin hydrochloride and canagliflozin in bulk drug is easy, accurate, precise and less time consuming.

Keywords: Metformin hydrochloride, Canagliflozin, RP-HPLC, Validation, Stability indicating.

Introduction

Metformin hydrochloride (Fig.1), an anti-diabetic drug is the first line oral pharmacotherapy for the cureof type 2 diabetes (1). It is chemically known as N,N-dimethyl-imido-dicarbon-imidicdiamide (2). It reduces the production of hepatic glucose. Hence, improves hyperglycemia (3). It also decreases the glucose production in the liver by activating the energy regulating enzyme Adenosine 5'-monophosphate-activated protein kinase (AMPK) which is considered a major mode of metformin action (1).

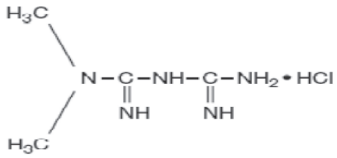

Fig.1. Structure of metformin hydrochloride

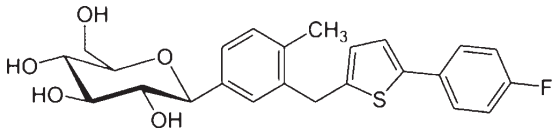

Fig. 2. Structure of canagliflozin

Canagliflozin (Fig.2) is a oral anti-diabetic agent which belongs to a newly developed class,ithas an inhibitory action on sodium-glucose co-transporter 2 (SGLT2).Itreceived approval by US FDA in March-2013 for treating the patients

having type-II diabetes. Canagliflozin is chemically named as (2S,3R,4R,5S,6R)-2-[3-[[5-(4-fluorophenyl)thiophen-2-yl]methyl]-4methylphenyl]-6(hydroxymethyl)oxane-3,4,5-triol(4, 5). SGLT2 is located in the proximal renal tubule and is majorly important for reabsorption of filtered glucose from its lumen, as SGLT2 is inhibited by canagliflozin the filtered glucose reabsorption decreases thereby lowering the kidney threshold for glucose and consequently increases the glucose excretion through urine (6).

Literature review for the simultaneous determination of metformin hydrochloride and canagliflozin by HPLC has revealed thatnot many analytical methods have been reported for the same (1, 6).By this study an easy, accurate, economic and efficient stability indicating Reverse phase-HPLC method has been developed for the simultaneous estimation of metformin hydrochloride and canagliflozin along with forced degradation studiesin bulk drug.

Materials and Methods

Chemicals and Reagents: Pharmaceutical grade reference standards for metformin hydrochloride and canagliflozin were obtained as gift samples byGlenmark Pharmaceuticals Ltd. (Sikkim, India) and MSN Life Sciences Pvt. Ltd. (Telangana, India) respectively. Methanol and acetonitrile used were of HPLC grade supplied by Finar chemicals limited, Ahmadabad. Ammonium acetate used was of analytical grade which was supplied by Spectrochem Pvt. Ltd., Mumbai. Ultra-clear water that has been used was from Millipore purification system.

***Instrumentation*:** Samples were analysed on Shimadzu HPLC (Kyoto, Japan) system controlled by LC solution software and equipped with a quaternary pump (LC-10 ADVP), Auto injector (SIL-10 ADVP), a photo diode array detector (SPD M-10A VP). The separation was done on a GraceSmart Reverse Phase18 (250 x 4.6 mm,5 µ) column.

Chromatographic Conditions: The mobile phase consisted of acetonitrile:10mM ammonium acetate buffer of pH 4.5 in the proportion of 45:55 (v/v). 0.77 gm of ammonium acetate was dissolved in 1 L of Milli-Q water and using 10 % glacial acetic acid the pH was adjusted to 4.5 ± 0.02. The resulting solution was undergone vacuum filtrationthrough a 0.45µm filter and later degassed in an ultrasonic bath for 15 minutes before use.The mobile phase was delivered with 1ml/min flow rate and the column temperature was 30ºC. The detection was carried out at 252 nm using PDA detector.The volume injected into the system was 20 microlitres having a total run time of 8 minutes.

Preparation of Standard Solution: 10mg each of metformin hydrochloride and canagliflozin working standard were accurately weighed and transferred separately into two 10 ml volumetric flasks and diluted up to the mark with methanol. From each volumetric flask, 1 ml of the stock solution was withdrawn and taken into a 10 ml volumetric flask and diluted to volume with mobile phase (MP). 1ml of the above solution was withdrawn and further diluted with mobile phase to the mark of 10ml flask to get working standard solution of 10µg/ml.

Results and Discussion

As a result, a typical chromatogram for the proposed method has been obtainedwhich is shown in Fig.3. The retention timesobserved for metformin hydrochloride and canagliflozin were found to be 4.00 min and 5.76 min respectively, having a resolution of 6.5.

Method Validation (7)*:*

System Suitability:It is carried out to evaluate the system suitability parameters (tailing factor, resolution, theoretical plates and relative standard deviation) for replicate injections The results obtained werewithin the limits and are shown in Table-1.

Specificity: It was performed to ensure that the response is due to single component only i.e. no co-elution exist between drug and excipients/ impurities. To perform this, a mixture of standard solution was prepared and peak purity was performed by PDA detector for metformin

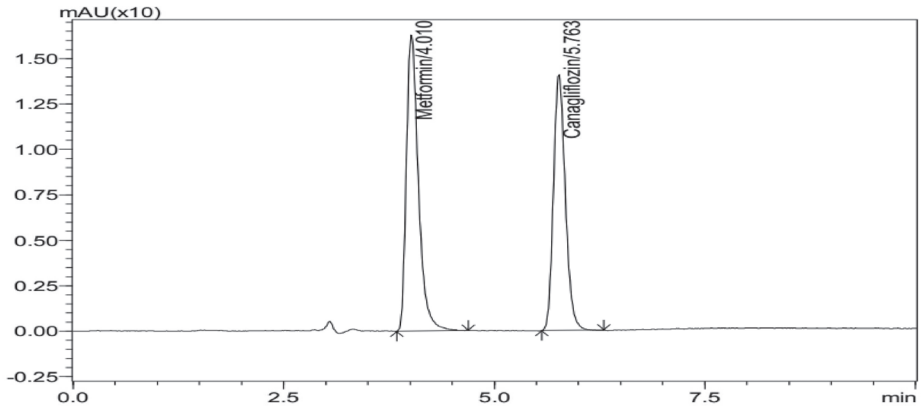

Fig.3. Optimised chromatogram of metformin hydrochloride and canagliflozin

hydrochloride and canagliflozin.The peak purity obtained was shown in Fig.4 and Fig.5.

Linearity and Range: A series of linearity solutions for the mixture of metformin hydrochloride and canagliflozin were prepared in the concentration range of 1-80 µg/ml. 20 µl of each standard was injected in triplicate and the results(chromatograms) were recorded for all the linearity standards under the optimized chromatographic conditions. The regression co-efficient for both the drugs were not less than 0.999, thus falls within the acceptance limits. The linearity graphs are shown in Figs. 6 and 7; and the results are tabulated in Table-2.

Limit of Detection (LOD) and Limit of Quantitation (LOQ): The LOD and LOQ of the developed method were determined from the standard deviation (SD) of the response and slope (m). The calculated values are tabulated in Table-3.

Accuracy: Accuracy studies have been conductedbased on the recovery of known amounts of analyteinorder to determine the proposed method accuracy by standard spiking method. The recovery of the analyte was calculated by spiking a noted amount of the

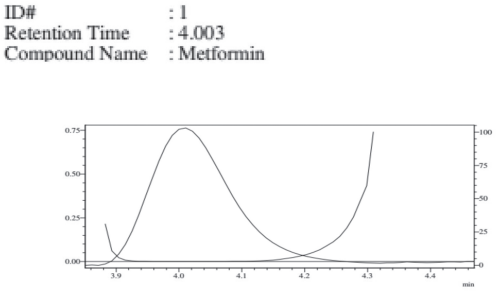

Fig. 4. Peak purity curve of metformin hydrochloride

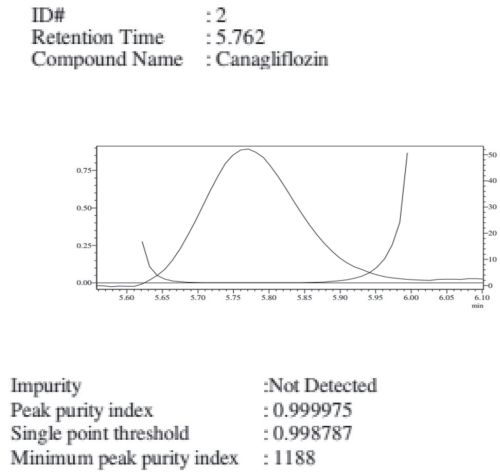

Fig. 5. Peak purity curve of canagliflozin

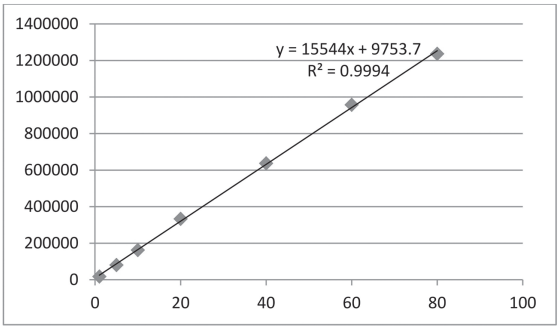

Fig. 6. Calibration curve of metformin hydrochloride

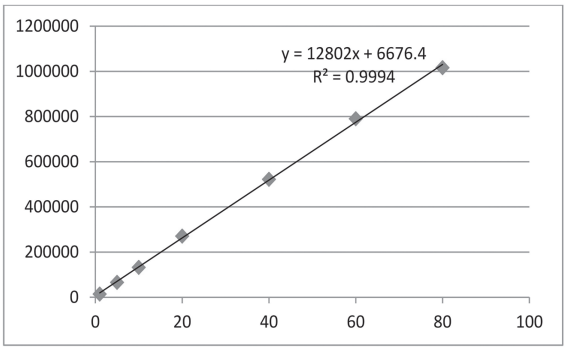

Fig. 7. Calibration curve of canagliflozin

standard drug to the pre analysed standard samples. Accuracy was performed at three known concentration levels of 80 %, 100 % and 120 % of standard concentration and sample solutionswere prepared in triplicate for each level. The % recovery was found to be within the acceptance criteria of 98-102%. The accuracy results are tabulated in Table4A and 4B.

Precision: Precision of the proposed method was carried out at three levels which were system precision, intermediate precision i.e. done in different days and different instrument and repeatability (method precision). The % RSD (result) was found to be less than 2.0% thus within the acceptance criteria. The precision results are tabulated in Table-5.

Robustness: Small variations were made in the analytical parameters of the method and robustness was studied by examining the peak area. The peak area obtained with each solution was measured and %RSD was calculated which was found to be less than 2.0%, thus within the acceptance criteria. Robustness results are tabulated in Table-6.

Forced Degradation Studies: These studies were conducted to indicate the stability indicating property of the developed method. The stressed samples of metformin hydrochloride and canagliflozin were then subjected toa peak purity test by using PDA detector. The conditions maintained for performing stress studies on metformin hydrochloride and canagliflozin are tabulated briefly in Table 7.

Acid Hydrolysis: From the stock solution (1000μg/ml) of metformin hydrochloride and canagliflozin, 1 ml was withdrawn and taken into a 10 ml volumetric flask and the volume was made up to the mark with 0.1M hydrochloric acid. The solution was then refluxed at 80 °C in a round bottom flask for 8 hours. The resultant solution was neutralized to pH 7.0 with counter base 0.1M sodium hydroxide and then diluted with mobile phase to 10μg/ml and then injected into the system. The chromatogram of acid degraded mixture after 4 hours on refluxing at 80°C is shown in Fig.8.

Alkali Hydrolysis: From the stock solution (1000 μg/ml) of metformin hydrochloride and canagliflozin, 1 ml was withdrawn and taken to a 10 ml volumetric flask and the volume was made up by using0.1 M sodium hydroxide. The solution was then refluxed at 80 °C in a round bottom flask for 8 hours. The resultant solution was neutralized to pH 7.0 with counter acid 0.1 M hydrochloric acid and then diluted with mobile phase to 10μg/ml and injected into the HPLC system.

The chromatogram of base degraded mixture after 2 hours on refluxing at 80°C is shown in Fig.9.

Oxidative Degradation: From the stock solution (1000μg/ml) of metformin hydrochloride and

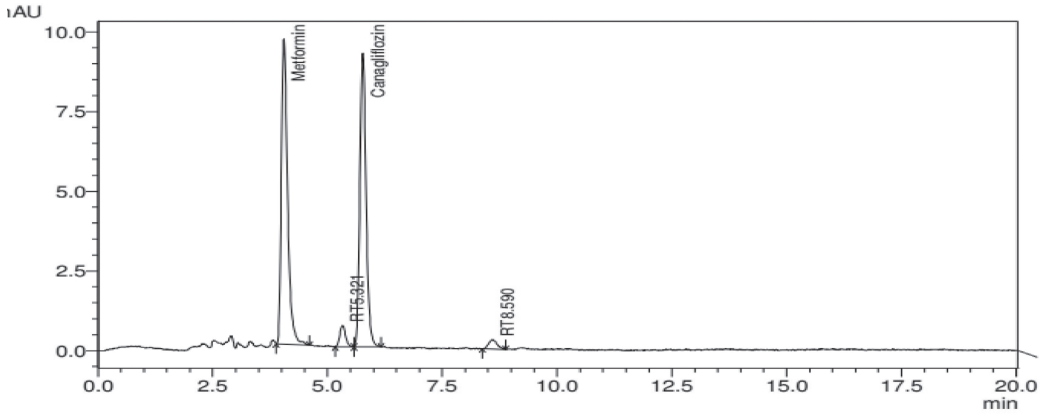

Fig.8. Chromatogram of acid degraded mixture after 4 hours (reflux at 80°C)

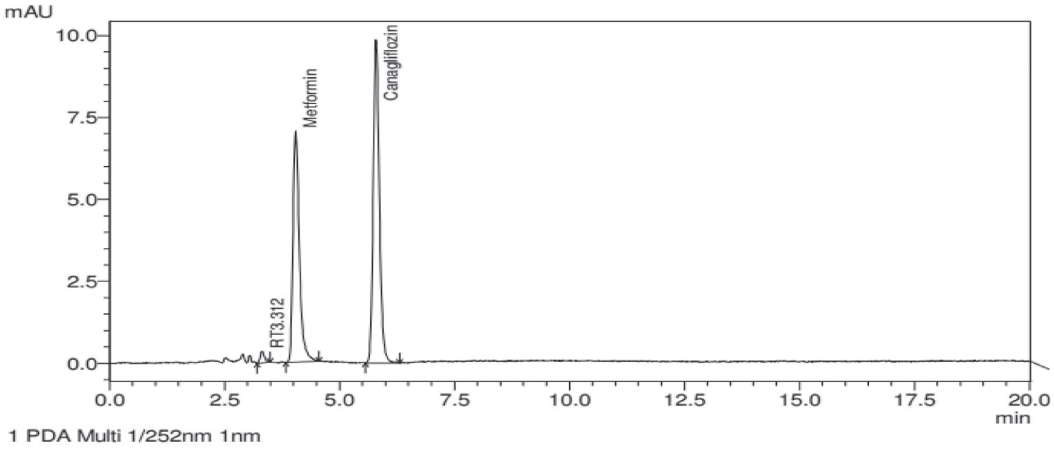

Fig.9. Chromatogram of base degraded mixture after 2 hours (reflux at 80°C)

canagliflozin, 1 ml was withdrawn and taken into a 10 ml volumetric flask and the volume was made up with 3 % v/v hydrogen peroxide solution. The resultant solution waskept aside at room temperature and diluted with mobile phaseto 10 µg/ml and injected into the system. The chromatogram of degraded mixture after 2 hrs at room temperature is shown in Fig.10.

***Thermal* Degradation:** Approximately 1 gm each of metformin hydrochloride and canagliflozin were placed separately in a petri dish and kept inside the hot air oven at 80°C for 24 hours. Samples were then collected, diluted with mobile phase to 10 µg/ml and injected into the system.

The chromatogram of mixture on exposure to heat at 24 hrs is shown in Fig,11.

Photolytic Degradation: A standard solution of 10µg/ml of metformin hydrochloride and canagliflozin in a 10 ml volumetric flask was kept outside exposed to direct sunlight for around 12 hours. The resultant solution was then injected into the system. The chromatogram of mixture on exposure to light after 12 hrs is shown in Fig.12.

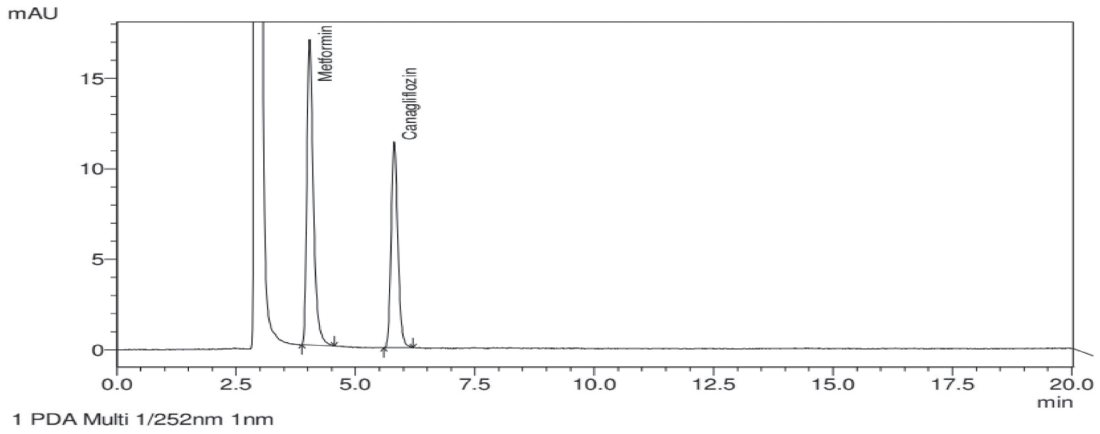

Fig.10. Chromatogram of degraded mixture after 2 hrs at room temperature

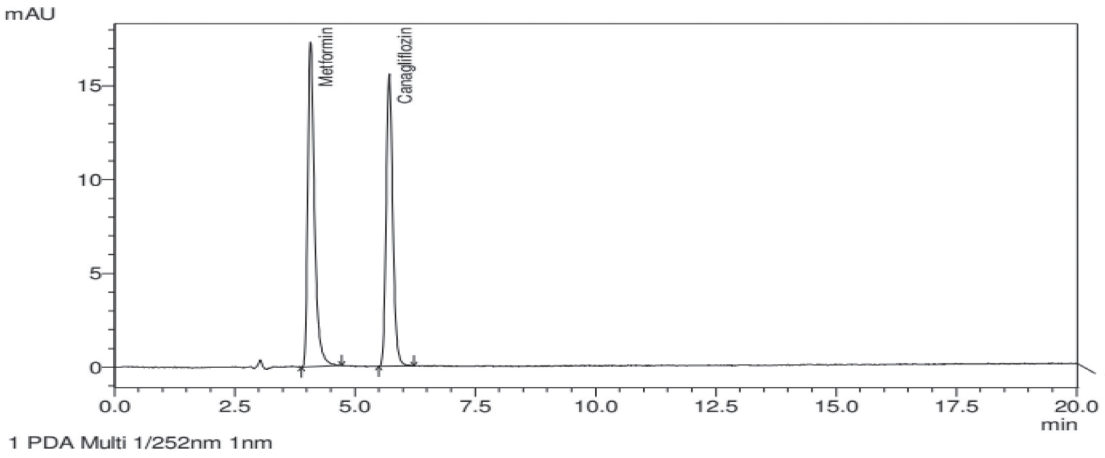

Fig.11. Chromatogram of mixture exposed to heat at 24 hrs

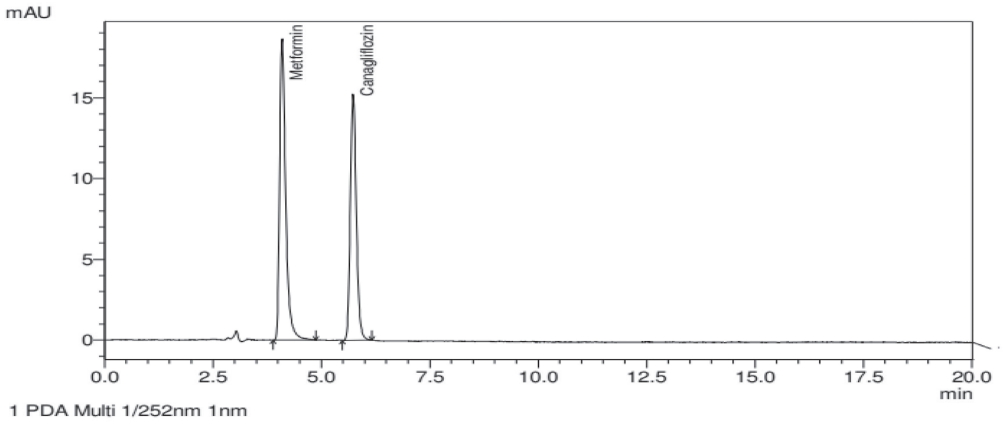

Fig.12. Chromatogram of mixture exposed to light after 12 hrs

Table 1. Summary of system suitability studies (n=6)

Parameter	Observation		Acceptance criteria
	Metformin HCl	Canagliflozin	
% RSD of retention time of 6 injections	0.10	0.10	Not more than 1.0
% RSD of area of 6 injections	0.32	0.30	Not more than 1.0
Plate count	3592.83	7264.17	More than 2000
Tailing factor	1.52	1.25	Not more than 2.0

Table 2. Data of linearity for metformin hydrochloride and canagliflozin

	Metformin hydrochloride	Canagliflozin
Regression equation	y = 15544x + 9753	y = 12802x + 6676
Correlation coefficient	0.999	0.999
Slope	15544	12802
Y-intercept	9753	6676

Table 3. Limit of detection and limit of quantitation

	Metformin HCl	Canagliflozin
LOD	0.134 μg/ml	0.124 μg/ml
LOQ	0.406 μg/ml	0.376 μg/ml

Table 4A. Accuracy results metformin hydrochloride

Spiked level	concentration (μg/ml)	Average Amount recovered(μg)	Average % recovery	%RSD
80%	18	17.95	99.39	0.10
100%	20	20.09	100.87	0.22
120%	22	21.83	98.65	0.31

Table 4B. Accuracy results canagliflozin

Spiked level	concentration (μg/ml)	Average Amount recovered(μg)	Average % recovery	%RSD
80%	18	17.93	99.19	0.27
100%	20	20.06	100.57	0.32
120%	22	22.01	100.08	0.11

Table 5. Precision studies of metformin hydrochloride and canagliflozin

Parameter	Drug	% RSD		
System Precision	Metformin hydrochloride	0.28%		
	Canagliflozin	0.27%		
Method Precision	Metformin hydrochloride	0.33%		
	Canagliflozin	0.39%		
Intermediate Precision: Different Days and Different Instruments	Day 1	Day 2	Inst 1	Inst 2
Metformin hydrochloride	0.33%	0.22%	0.31%	0.75%
Canagliflozin	0.39%	0.28%	0.27%	0.49%

*Average of 6 injections

Table 6: Robustness results of metformin hydrochloride and canagliflozi**n**

Parameter	Changed condition	% RSD	
		Metformin hydrochloride	Canagliflozin
pH [±0.2 units]	pH 4.3	0.36	0.33
	pH 4.5	0.49	0.34
	pH 4.7	0.30	0.33
Wavelength[±2 nm]	250 nm	0.44	0.10
	252 nm	0.45	0.39
	254 nm	0.31	0.25
Flow rate [±10%]	0.9 ml/min	0.27	0.25
	1.0 ml/min	0.30	0.31
	1.1 ml/min	0.25	0.18
Mobile phase [±2units]	43:57	0.45	0.42
	45:55	0.52	0.32
	47:53	0.37	0.30
Column oven temperature [±5°C]	25°C	0.24	0.16
	30°C	0.42	0.22
	35°C	0.23	0.21

*Average of 6 injections

Conclusion

The developed stability indicating assay method is found to be easy, accurate, sensitive, specific and rapid for the simultaneous estimation of metformin hydrochloride and canagliflozin and was validated in terms of accuracy,linearity, precision, limits of detection (LOD) and quantification (LOQ)according to ICH Q2(R1) guidelines. Stress studies were performed for the drug substance under acidic, alkaline, photolytic,oxidative and thermal conditions using the above optimised method. In all the conditions, the drug peaks were separated from the degradation product peak. This method can thus be applied for routine normalanalysis and stability testing of metformin hydrochloride and canagliflozin in pharmaceutical formulation like tablets and in bulk.

Table 7. Forced degradation study results of metformin hydrochloride and canagliflozin

Type of degradation	Stress Conditions	Degradation time	% of degradation observed		Peak purity
			Metformin hydrochloride	Canagli flozin	
Acid hydrolysis	0.1 M Hydrochloric acid at 80°C reflux	4 hrs	12 %	7 %	Passed
Alkali hydrolysis	0.1 M sodium hydroxide at 80 °C reflux	2 hrs	32 %	5 %	Passed
Oxidative degradation	3 % v/v Hydrogen peroxide at room temperature	2 hrs	13 %	15 %	Passed
Thermal degradation	Inside hot air oven at 80 °C	24 hrs	7 %	1 %	Passed
Photolytic degradation	Exposed to direct sunlight	12 hrs	2.7 %	2.6 %	Passed

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Effect of Bulking Agents, Type of Cultures and Compression Pressures on Functional Properties of Probiotic and Starter Culture Tablets

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Abstract

In the current study freeze dried cultures of *Streptococcus thermophilus* MTCC 5460, *Lactobacillus helveticus* MTCC 5463, *Lactobacillus rhamnosus* MTCC 5462 and *Lactobacillus delbrueckii* subsp. *bulgaricus* NCIM 2358 were used for preparation of tablets to be used as either inocula for product preparation or as dietary supplement. Maltodextrin and spray dried lactose as bulking agents were compared for preparation of active ingredients. Selection of bulking agent was based on the viability, activity, micromeritic properties and stability of the active ingredients prepared. Active ingredients of the cultures were mixed with other excipients and subjected to direct compression at different compression pressures *viz.*, low (1-2 kg/cm^2), medium (3-4 kg/cm^2) and high (5-6 kg/cm^2) to prepare tablets. The tablets were analyzed for hardness, disintegration and viability. Viability and activity of the freeze dried cultures were not significantly affected by the bulking agents indicating a good compatibility of cultures and bulking agents used. Between the two active ingredients containing maltodextrin or spray dried lactose as bulking agent, spray dried lactose showed better flow properties, hence it was selected. A significant ($P<0.05$) increase in the hardness and disintegration time of tablets as well as a significant ($P<0.05$) decrease in the viability of all four cultures were seen when the compression pressure was increased from 1-2 kg/cm^2 to 5-6 kg/cm^2. Hence a compression pressure of 1-2 kg/cm^2 was selected for preparation of culture tablets.

Keywords

Lactobacillus helveticus MTCC 5463, probiotic tablets, starter culture, compression pressure, viability

Introduction

Lactic acid bacteria (LAB) as starter cultures are widely used in dairy industry for production of a variety of fermented milk products. Selected strains of LAB have found application as either probiotics or synbiotics and are used as food ingredient or dietary supplements (1-3). The increasing application of starter cultures and probiotics demand them to be made available to the consumers in dosage forms, which can provide ease of handling, ease of addition to food, precise dosage and functionality for specific application and long term preservation. Current marketing strategies of probiotics dispense them as ingredients in foods, nutritional supplements and as pharma products (3). These probiotic products can be taken either directly or along with food and beverages. Freeze dried powder of starter cultures and/or probiotic cultures in dry form are widely used for fermented product preparation or for preparation of various dosage forms such as tablets and capsules. In such dosage forms, live freeze dried bacterial cells are blended with certain protective as well

as bulking agents. Additionally, certain excipients are also incorporated to facilitate ease of preparation and to get desired dosage form characteristics such as flow properties in case of tablets. In such cases, the functionality of the dosage preparations are largely defined by the survival and activity of the starter cultures and/ or probiotic cultures which need to be maintained throughout the shelf life of the dosage form. The processing parameters such as compression pressure applied and the type and characteristics of the bulking agents as well as the excipients used in the tablet formulation has a great influence on survival and functionality of the cultures (4-7). Taking these important aspects into consideration, the present research work was carried out to optimize the active ingredient preparations for probiotic/ starter cultures as well as to select the most suitable compression pressure for preparation of culture tablets to be used as either inocula or food supplement.

Materials and Methods

***Microbial Cultures*:** Starter cultures *viz*., *Streptococcus thermophilus* MTCC 5460 and *Lactobacillus delbrueckii* subsp. *bulgaricus* NCIM 2358 and probiotic cultures *viz*., *Lactobacillus helveticus* MTCC 5463 and *Lactobacillus rhamnosus* MTCC 5462 were used in the current study. All the cultures are indigenous isolates obtained from Dairy Microbiology Department of SMC College of Dairy Science, Anand Agricultural University, Anand, Gujarat, India except NCIM 2358 which is obtained from National Dairy Research Institute, Karnal, India. The probiotic cultures are thoroughly studied for their probiotic potential (8, 9). All the cultures were propagated in sterilized reconstituted skim milk (12% Total solids) by incubation at 37°C for 24 h and stored at 5 ± 2 °C. During the course of study, prior to their use, the cultures were given three successive transfers in the whey medium to activate them.

Chemicals: Analytical grade chemicals were used during the entire study. L-Ascorbic acid was purchased from Qualigens Chemicals Pvt. Ltd., Mumbai. Maltodextrin was procured from Loba Chemie Pvt. Ltd., Mumbai, India and cryoprotectant Glycerol was supplied by Glaxosmithkline Pharmaceuticals Ltd., Mumbai. Skimmed Milk Powder (SMP) used for the preparation of suspending medium belonged to Sagar brand and was procured from the local market. Other excipients used in the study included Spray dried lactose and Superstarch 200, supplied by DFE Pharma Pvt. Ltd., Klever Strasse; Crospovidone/ Sodium Starch Glycolate/ Cross carmelose sodium, Polyvinyl pyrrolidione K-30, Magnesium stearate and talc supplied by S. d. Fine Chem. Ltd., Mumbai, India.

Preparation of Freeze Dried Cultures and their Active Ingredients (AI): Freeze dried powder of the cultures were prepared using protocol optimized by Jani *et al.* (10). The active cultures were inoculated in the whey medium at 2% rate and grown at 37°C for 24 h. The cells were harvested by centrifugation at 6000 rpm at 4 °C for 20min. Cell pellets were washed twice with saline water (0.85% NaCl solution). The cells were then suspended in 12 % reconstituted skim milk added with 1% Glycerol (as cryoprotectant). The contents were mixed thoroughly and distributed in glass petriplates. The suspension was frozen at -20°C for overnight and freeze dried using freeze dryer model Virtis genesis 25XL. During the entire drying process, vacuum of 100 millitorr was maintained. Temperatures in the drying chamber were gradually increased from -40 °C to 30 °C over a period of 15-16 h looking to the process of drying. The freeze dried cultures were individually mixed with reducing and bulking agents for preparing the active ingredients (AI) to be used for dosage forms. L-Ascorbic acid and spray dried lactose were used as the reducing and bulking agent respectively. The rate of culture, reducing agent and bulking agent in the AIs were fixed as 20, 20 and 60% (w/w) respectively based on study done by Panchal *et al*. (11). The AIs were studied for culture–excipient compatibility and micromeritic characteristics.

***Estimation of Viability, Activity and Micromeritic Properties*:** Serial dilutions of the

active ingredients were made using 2% peptone water as dilution blank. For preparing the initial dilution 0.1 g powder was reconstituted in peptone water and incubated at 37°C for 2 h to recover cell injuries. Subsequent dilutions (as per requirement) were then prepared in peptone water and appropriate dilutions were pour plated using respective selective agar medium. MRS medium was used for lactobacilli and M17 medium was used for streptococci. Once the initial agar layer was set, a second layer (5-8 ml) of the same medium was made to maintain facultative anaerobic conditions. The plates were then incubated at 37±2°C for 72 h. Colony counts were taken with the help of colony counter and the count was expressed as log (cfu/g) (12). To test the activity, all four freeze dried cultures and their AIs were checked for their ability to form curd. The inoculation rate was 0.1g/100ml milk. After inoculation of the samples, the skim milk flasks were incubated at 37°C for overnight (15h). The curd was analyzed for titratable acidity, pH and viable count. Active ingredients (AI) were evaluated for bulk density, tapped density, angle of repose, Carr's index and Hausner's ratio to evaluate micromeritic properties (13).

***Microbiological Analysis*:** Eleven grams of curd sample was aseptically weighed and transferred to 99ml phosphate buffer dilution blank to obtain 1:10 dilution. Subsequently, 1 ml of above dilution was used for making further dilutions in 9 ml phosphate buffer tubes. Suitable dilutions were prepared and poured in a set of sterile petri dishes in duplicates.

***Stability of Active Ingredients*:** Accurately weighed amounts of active ingredients were exposed to relative humidity (RH) conditions of 10, 52, 75 and 92% obtained by using saturated aqueous solutions of lithium chloride, magnesium nitrate, sodium chloride and potassium nitrate respectively, in sealed desiccators at temperature of 25°C. After 7 days, samples were analyzed for moisture gain and classified on the basis of hygroscopicity (14).

***Preparation of Culture Tablets by Direct Compression*:** All the excipients were passed through 80 mesh sieve. Required quantities of AIs and excipients except lubricant and glidant were mixed thoroughly in a double cone blender. The powder blend was mixed with lubricant glidant mixture. This powder mixture was compressed in ten station rotary tablet machine (Rimek, RSB4-1, Karnavati Engg. Pvt. Ltd., Ahmedabad, India) with flat faced punches of diameter 5mm at different compaction forces *viz*., low (1-2 kg/cm^2), medium (3-4 kg/cm^2) and high (5-6 kg/cm^2) (7). The tablets were analyzed for hardness, disintegration time and microbiological assay to assess the effect of compression pressure on the viability of cultures as well on disintegration time.

***Analysis of Tablets for Disintegration, Hardness and Viability*:** For estimating the disintegration time**,** the tablets were put in the water maintained at 37°C and the time of complete dissolution was noted. Hardness of tablets was measured using Monsanto hardness tester. For estimating the viability of tablets, serial dilutions of the tablets were made using 2% peptone water as dilution blank. For preparing the initial dilution one tablet was dissolved in 10ml of peptone water. Subsequent dilutions (as per requirement) were then prepared in peptone water and appropriate dilutions were pour plated using respective selective agar medium. Once the initial agar layer was set, a second layer (5-8 ml) of the same medium was made to maintain facultative anaerobic conditions. The plates were then incubated at 37±2°C for 72 h. Colony counts were taken with the help of colony counter and the count was expressed as log cfu/tablet. For checking the activity of the tablets, one tablet was put in 100ml sterile reconstituted skim milk (12%Total solids) and dissolved completely. It was then incubated at 37±2°C for overnight (15h). The curd formed was evaluated for sensory characteristics by expert panel of judges using nine point hedonic scales.

***Statistical Analysis*:** The values of each attribute under study were subjected to statistical analysis using Completely Randomized Design with equal

number of observations using the model proposed by Steel and Torrie (15).

Results and Discussion

***Viability and Activity of Freeze Dried Cultures*:** The average viable counts of fresh freeze dried *Streptococcus thermophilus* MTCC 5460, *Lactobacillus helveticus* MTCC 5463, *Lactobacillus rhamnosus* MTCC 5462 and *Lactobacillus delbrueckii* subsp. *bulgaricus* NCIM 2358 were found to be 10.57±0.08, 10.73±0.12, 10.79±0.15 and 10.67±0.20 log cfu/g respectively. These counts were in line with the viability of strains of lactobacilli and streptococci observed by earlier workers (10, 11, 16). The average rate of acid development in sterile reconstituted skim milk upon inoculation of freshly prepared freeze dried cultures at the rate of 0.1g/100ml milk and incubation at 37°C for overnight (12-14h) is shown in Table 1. A significant ($P < 0.05$) difference in the rate of acid development was observed among the four cultures, which was expected keeping in mind the variation in the milk fermenting ability of the four cultures. Likewise a significant ($P < 0.05$) difference was observed for pH of the curds and the respective culture counts. The curd obtained was good and uniform quality. The set curds were sufficiently firm to hold their shape when poured. There was no whey separation and the curds were organoleptically acceptable. However, as expected, the coagulation time by freeze dried cultures was longer than otherwise traditionally propagated active liquid cultures.

Table 1. Viability and activity of freeze dried cultures

Cultures	Viability (log cfu/g)	Acidity (% Lactic Acid)	pH	Count in Curd (log cfu/g)
Streptococcus thermophilus MTCC 5460	10.57±0.08	0.82±0.07^{a}	4.46±0.07^{b}	9.25±0.03^{a}
Lactobacillus helveticus MTCC 5463	10.73±0.12	1.77±0.06^{b}	3.34±0.10^{a}	9.55±0.03^{c}
Lactobacillus rhamnosus MTCC 5462	10.79±0.15	1.63±0.22^{b}	3.44±0.15^{a}	9.35±0.22^{ab}
Lactobacillus delbrueckii subsp. *bulgaricus* NCIM 2358	10.67±0.20	0.78±0.04^{a}	4.47±0.07^{b}	9.44±0.10^{bc}
CD (0.05)	NS	0.19	0.17	0.19

Each observation is mean±SD of four replications; NS= Not Significant
$^{a\text{-}c}$ Superscript letters following numbers in the same column denote significant difference ($p<0.05$)

Table 2. Viability and activity of AI1*

Active ingredients of	Viability (log cfu/g)	Acidity (% Lactic Acid)	pH	Count in Curd (log cfu/g)
Streptococcus thermophilus MTCC 5460	9.17±0.09^{a}	0.71±0.03^{a}	4.84±0.36^{b}	9.13±0.06^{a}
Lactobacillus helveticus MTCC 5463	9.80±0.13^{b}	1.00±0.14^{b}	4.31±0.30^{a}	9.37±0.03^{b}
Lactobacillus rhamnosus MTCC 5462	9.79±0.08^{b}	1.02±0.02^{b}	4.28±0.10^{a}	9.23±0.11^{a}
Lactobacillus delbrueckii subsp. *bulgaricus* NCIM 2358	9.71±0.11^{b}	0.81±0.01^{a}	4.54±0.02^{ab}	9.18±0.06^{a}
CD (0.05)	0.16	0.11	0.37	0.11

*AI1 =Freeze dried culture : Ascorbic acid: Maltodextrin (20:20:60)
Each observation is mean±SD of four replications
^{a,b} Superscript letters following numbers in the same column denote significant difference ($p<0.05$)

***Viability and Activity of Active Ingredients*:** Freeze dried cultures and their preparations are meant for longer period of storage and hence maintaining the viability and activity of the cultures in the preparations during the storage period is of prime importance for successful application of the same. If the freeze dried cultures are stored as it is, the viable count may go down and its efficiency to coagulate milk reduces significantly. Hence, it is advisable to incorporate reducing agents and bulking agents for maintaining better viability and activity of freeze dried cells. At the same time, compatibility of these reducing and bulking agents with the cultures in terms of their viability and activity is also important. The incorporation of bulking agents improve the bulk of the lyophilized product, provide an adequate structure to the cake, improve physical characteristics such as flow ability, ease of handling, non-hygroscopicity, etc of the freeze dried preparation which are essential during dosage form making. The incorporation of excipients affect the micromeritic properties of active ingredients such as angle of repose, carr's index and hausner's ratio which are considered important for the flow properties and compaction behavior during dosage form making. The relationship between flow, angle of repose and carr's index is interpreted as per Indian Pharmacopeia (IP)/United States Pharmacopeia (USP). L-Ascorbic acid as reducing agent and maltodextrin and spray dried (SD) lactose as bulking agents were used for preparation of active ingredients (AI) based on the results of earlier study conducted by Panchal *et al*. (11) and other preliminary trials. The viability and activity of the active ingredients prepared using maltodextrin are shown in Table 2.

A significant ($P < 0.05$) difference was observed in the viable counts in the active ingredient preparations of all the four cultures. The acidity, pH and viable counts in curds obtained also showed significant ($P < 0.05$) difference when maltodextrin was used as bulking agent. This may be due to the inherent difference in the fermenting ability of the four cultures used in the study. Andersen *et al*. (17) showed that maltodextrins with a low dextrose equivalent value better preserved the acidifying activity of *Streptococcus thermophilus* compared to maltodextrins with a high dextrose equivalent value. Among the four cultures, the highest count was observed for MTCC 5463 (9.80±0.13 log cfu/g) and the lowest for MTCC 5460 (9.17±0.09). Muller *et al*. (18) reported the protective effect of maltodextrin during the reconstitution of the probiotic powder before its usage. Panchal *et al*. (11) used maltodextrin as bulking agent and ascorbic acid as reducing agent to stabilize probiotic vaginal strain *Lactobacillus helveticus* MTCC 5463 during storage of freeze dried preparation.

The viability and activity of the active ingredient preparation where maltodextrin was replaced with spray dried lactose is shown in Table 3. A significant ($P < 0.05$) difference was observed in the viable counts of the preparation, curd acidity, pH and count among the four cultures. Highest viable count was observed for MTCC 5462 (9.89 ± 0.09 log cfu/g) and the lowest for MTCC 5460 (9.41 ± 0.20 log cfu/g). Zarate and Nader-Macias (19) used lactose in combination with skim milk and ascorbic acid to stabilise probiotic vaginal strains during freeze-drying and storage. Between the two active ingredients, the viable counts were found to be higher in case of AI using spray dried lactose. No significant differences were seen for other parameters such as curd acidity, pH and count.

***Micromeritic Properties of Active Ingredients*:** A comparative evaluation of the micromeritic properties of the active ingredients prepared using maltodextrin and spray dried lactose is shown in Figures 1, 2 and 3. The results showed that the values for angle of repose and Carr's index differed significantly ($P < 0.05$) between the four cultures in case of AI1 and AI2. The values of angle of repose, Carr's index and Hausner's ratio indicated significant ($P < 0.05$) difference between the micromeritic properties of two active ingredients. For AI1, the values indicated poor to very poor flow property. While for AI2, the

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values indicated better flow properties. Good flow characteristics are a pre requisite for preparation of dosage forms such as tablets. Hence spray dried lactose was selected as bulking agent for the study. Spray dried lactose enables direct compression of formulations in a simple manufacturing process. The narrow particle size distribution is an important factor which affects the appropriate flow properties of spray dried lactose (20).

***Stability Study*:** Active ingredients containing spray dried lactose for all four cultures were further studied for stability at different relative humidity conditions for 7 days at 25°C. The result of the stability study is depicted in Figure 4. The moisture uptake was found to be less than 20% (as per ICH guidelines) in case of all active ingredients except for MTCC 5460 which was slightly higher (20.63). Bora *et al.* (4) has carried out preformulation studies of probiotic *Bacillus*

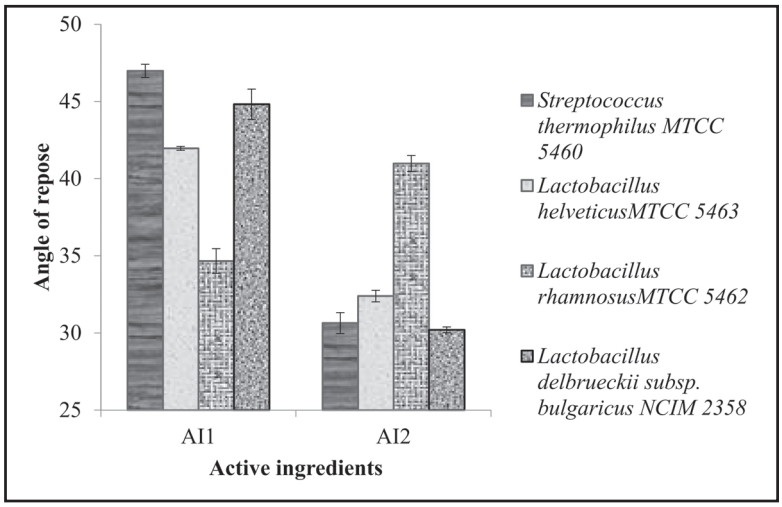

Fig. 1. Comparative evaluation of angle of repose of active ingredients

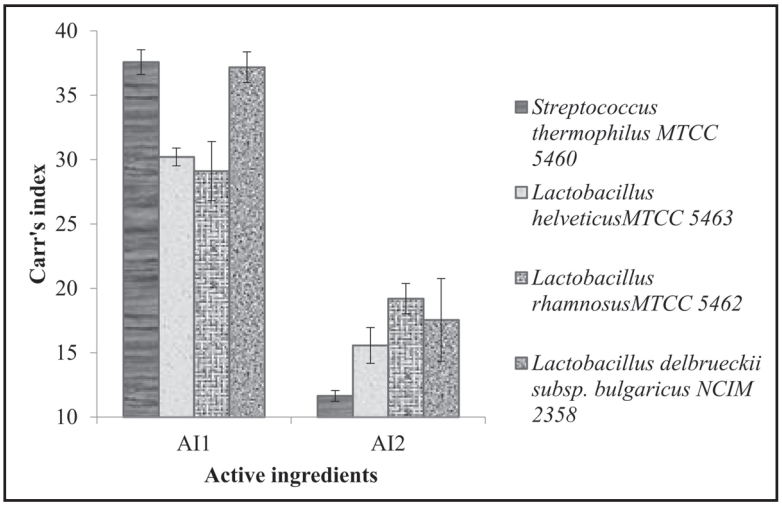

Fig. 2. Comparative evaluation of Carr's index of active ingredients

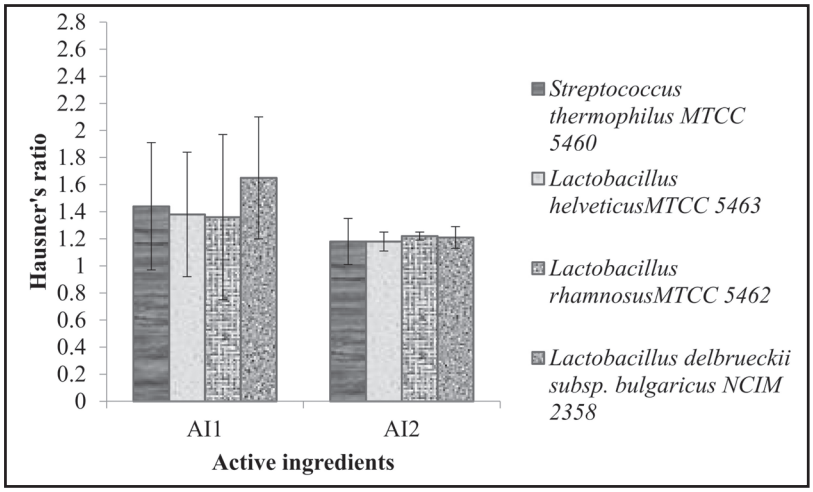

Fig. 3. Comparative evaluation of Hausner's ratio of active ingredients

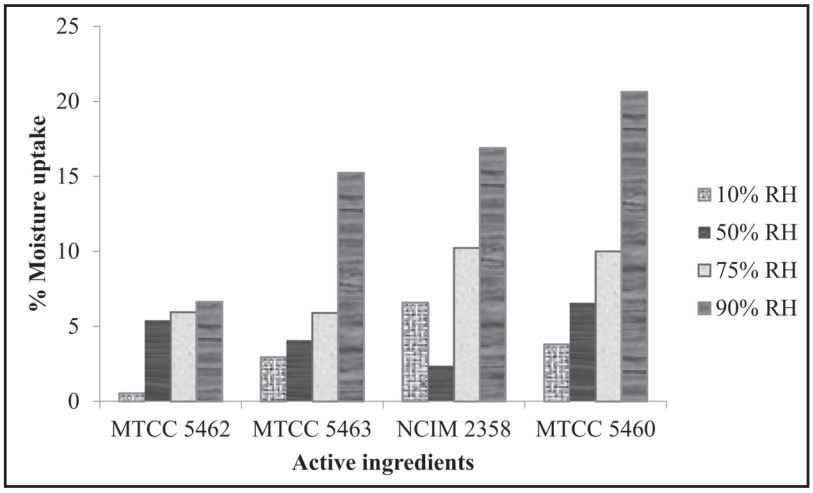

Fig. 4. Stability study of active ingredients containing spray dried lactose

coagulans spores to aid designing of stable formulations. They have studied the excipient compatibility studies of probiotic *Bacillus coagulans* spores and found reduced assay with citric acid monohydrate, meglumine and sodium starch glycolate. The loss of activity seemed to be related to the moisture uptake, free and bound water present in the bulk. The current study results indicated the non hygroscopic nature of active ingredients used and their suitability for preparation of dry dosage forms.

Effect of Compression Pressure on Hardness, Disintegration Time and Viability of Tablets: Preparation of dosage forms such as tablets require compression of all ingredients in a tablet press. In direct compression process active ingredient is blended with a variety of excipients, subsequently lubricated and directly compressed into a tablet. The formulation used for tablets preparation in the current study is shown in Table 4. Formulations varied with respect to type of active ingredients (100mg) only. The effect of

different levels of compression pressure on hardness, disintegration time and viability of tablets is shown in Table 5. A significant ($P < 0.05$) increase in the hardness (n=6) and disintegration time (n=3) of tablets was observed for all four cultures when the compression pressure was increased. This increase was found to be higher (Mean = 3.56) for tablets containing *Lactobacillus helveticus* MTCC 5463 compared to other three culture tablets. Among the cultures, the increase in disintegration time of tablets was found to be not significant. But the interaction effect was again significant (*P<0.05*). The disintegration time of a tablet depends on the hardness of tablet, the kind and amount of disintegrating agents used and how it acts on the tablet formulation (21). Sodium starch glycolate used in the formulation is a superdisintegtant. The extent of cross-linking and degree of substitution in sodium starch glycolate allows for rapid water uptake by the polymer without the formation of a viscous gel (22). Compression pressure had a significant effect ($P < 0.05$) on the viability of cultures in the tablets. Viability decreased linearly with increasing level of compression pressure. This decrease in viability of cultures was found to be not significant among the cultures. The interaction effect was also not significant.

A number of studies have indicated the effect of compression pressure applied on viability of culture tablets. Fazeli and coworkers (23) reported nearly 1 log cycle reduction in the assay values of tablets of *Lactobacillus acidophilus* after compaction. Durand and Panes (24) demonstrated that different species of probiotic bacteria had different levels of resistance to compaction pressure. The study results of Klayraung *et al*. (5) showed that the proportion of matrix forming excipients in tablets and the compression force affected the properties of probiotic tablets in terms of tensile strength and disintegration as well as the survival of the bacteria. Brachkova *et al*. (25) produced several formulas of mini tablets with or without microcrystalline cellulose and inulin (2.5 mm diameter) and several strains of *Lactobacillus* by applying compaction forces of 1, 2, and 5 kN and reported a decrease of less than 2 log units in the viability of probiotic bacteria. Nagashima *et al*. (21) in their study on development of effervescent tablets with probiotics *Lactobacillus acidophilus* and *Saccharomyces boulardii* reported a decrease in concentration of microorganisms with an increase in hardness when the compression force reached over 20 N. e Silva *et al*. (6) in their research on development of probiotic tablets using microparticles studied the effect of compaction force on viability of the probiotic strain *L. paracasei* L26. Among the compaction forces tested 9.8, 19.6, 29.4, and 39.2 kN, a decrease of 1 log cycle was observed after the compaction with 9.8-kN force. However, the number of viable cells for different compaction forces was of the same order of magnitude showing no increase of detrimental effects for compaction forces higher than 9.8 kN ($p>0.05$).

In the current study, the percent survival of cultures in the tablets decreased significantly (*P < 0.05*) with increasing compression pressure (Figure 5). The survival was found to be nearly 90% (mean=89.75) for all four cultures when the compression pressure applied was 1-2 kg/cm^2. This survival rate decreased from 90% to 76.76% and to 71.75% when the pressure was increased from 1-2 kg/cm^2 to 3-4kg/cm^2 and subsequently to 5-6 kg/cm^2. Between the cultures the difference in survival was found to be non significant. Depending on the pressure applied, the compression of cells may cause damage to the cell walls and membranes or even loss of viability. It is clear that under mechanical stress some cells cannot tolerate such compression. Initially, the increase in the force applied will primarily damage the cell wall and when such pressure is further increased, it will also reach the cell membrane. Therefore, it has been observed that cellular viability decreases almost linearly with the applied compression force (26). Maggi *et al*. (27) also verified a reduction in viability in different strains of lactobacillus during tablet production with other lyophilized strains. Bora *et al*. (4) observed a decline in spore formation in *Bacillus coagulans*

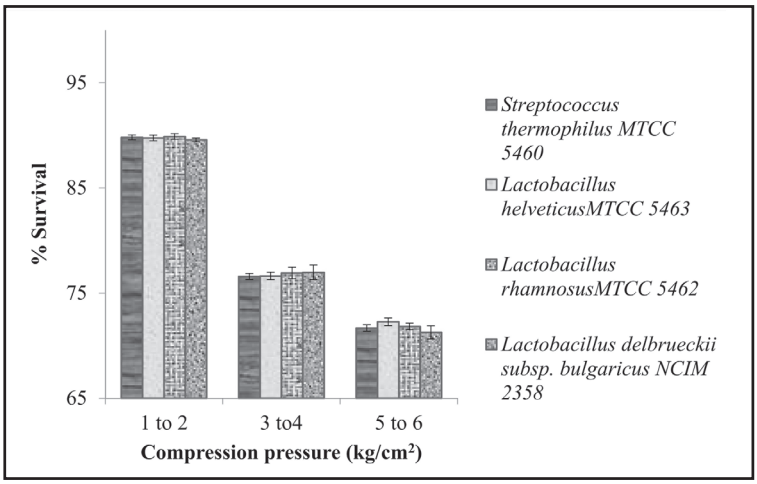

Fig. 5. Percent survival of cultures in tablets made using different compression pressures

Table 3. Viability and activity of AI2*

Treatments (Cultures)	Viability (log cfu/g)	Acidity (% Lactic Acid)	pH	Count in Curd (log cfu/g)
Streptococcus thermophilus MTCC 5460	9.41 ± 0.20[a]	0.70 ± 0.05[a]	4.75 ± 0.21[c]	9.18 ± 0.07[a]
Lactobacillus helveticus MTCC 5463	9.85 ± 0.09[b]	0.89 ± 0.03[b]	4.49 ± 0.03[b]	9.40 ± 0.06[b]
Lactobacillus rhamnosus MTCC 5462	9.89 ± 0.09[b]	1.09 ± 0.01[c]	4.14 ± 0.01[a]	9.17 ± 0.09[a]
Lactobacillus delbrueckii subsp. *bulgaricus* NCIM 2358	9.76 ± 0.12[b]	0.84 ± 0.02[b]	4.53 ± 0.01[b]	9.18 ± 0.[07a]
CD(0.05)	0.20	0.05	0.16	0.11

*AI2 = Freeze dried culture : Ascorbic acid: Spray dried lactose (20:20:60)
Each observation is mean±SD of four replications
[a-c] Superscript letters following numbers in the same column denote significant difference ($p<0.05$)

Table 4. Formulation used for preparation of tablets

Ingredients	Quantity (mg)
Active ingredient*	100
Spray Dried Lactose	195
Super Starch 200	100
Sodium Starch Glycolate	50
Talc	20
Magnesium Stearate	15
Total weight of Tablet (mg)	480

*Formulations varied with respect to type of active ingredients.

probiotic with an increase in the compression force, indicating that survival also depends on the probiotic species and on the excipients used in the formulation. Overall, our study results were found in agreement with that of the previously discussed research works where it was reported that the compression pressure had a significant effect on the hardness, disintegration time and viability of probiotic tablets. An increased compression pressure lead to tablets with increased hardness, longer disintegration time and decreased viability.

Table 5. Effect of different compression pressures on hardness, disintegration time and viability of tablets

Active ingredients of	Compression Pressure (kg/cm^2) 1-2	3-4	5-6
	Hardness		
Streptococcus thermophilus MTCC 5460	1.32 ± 0.14	3.34 ± 0.14	5.40 ± 0.13
Lactobacillus helveticus MTCC 5463	1.44 ± 0.17	3.23 ± 0.1	5.41 ± 0.12
Lactobacillus rhamnosus MTCC 5462	1.26 ± 0.12	3.14 ± 0.06	5.23 ± 0.03
Lactobacillus delbrueckii subsp. *bulgaricus* NCIM 2358	1.26 ± 0.12	3.16 ± 0.02	5.23 ± 0.03
CD (0.05) A=0.09, P=0.08; AxP=NS			
	Disintegration time (minutes)		
Streptococcus thermophilus MTCC 5460	1.31 ± 0.17	2.10 ± 0.14	3.14 ± 0.05
Lactobacillus helveticus MTCC 5463	1.24 ± 0.13	2.23 ± 0.06	3.13 ± 0.09
Lactobacillus rhamnosus MTCC 5462	1.13 ± 0.07	2.10 ± 0.04	3.21 ± 0.09
Lactobacillus delbrueckii subsp. *bulgaricus* NCIM 2358	1.19 ± 0.04	2.13 ± 0.06	3.07 ± 0.05
CD (0.05) A= NS, P=0.06; AxP=0.12			
	Viability (log cfu/tablet)		
Streptococcus thermophilus MTCC 5460	8.92 ± 0.02	7.61 ± 0.04	7.12 ± 0.02
Lactobacillus helveticus MTCC 5463	8.93 ± 0.03	7.63 ± 0.04	7.19 ± 0.03
Lactobacillus rhamnosus MTCC 5462	8.95 ± 0.01	7.65 ± 0.03	7.15 ± 0.04
Lactobacillus delbrueckii subsp. *bulgaricus* NCIM 2358	8.92 ± 0.01	7.66 ± 0.08	7.09 ± 0.05
CD (0.05) A= NS, P=0.03; AxP= NS			

Each observation is mean±SD of four replications; NS= Not Significant

Conclusion

The study results clearly indicate the significant effect of bulking agents, cultures and compaction forces on properties of probiotic and starter culture tablets. Also the effect of compression pressure is largely decided by the kind and proportion of matrix forming excipients in tablets. Hence along with proper compression pressure, proper selection of the matrix forming excipients is vital for survival of bacterial cultures in dosage forms such as tablets. As the functionality of such culture tablets are greatly depended on the viability and activity of the cultures used, strain to strain variation should be taken into account while optimizing the various parameters.

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Detection of Adulteration in Uncooked Kashmiri Mutton Product (Rista) with Cattle and Buffalo Meat

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Abstract

The Jammu and Kashmir is a state in India where people are predominantly non-vegetarian, more so in Kashmir valley where meat and meat products are consumed since ages as exemplified by the world famous Kashmiri cuisine, *Wazwan*, with *Rista* being one of the predominant dishes. The fraudulent adulteration of superior quality meat with inferior quality meat is a practice that has been observed all over the world. The meat industry in India is largely unorganized; hence adulteration or substitution of meat in meat products is likely to be practiced. In view of such likely problem in indigenous meat products of Kashmiri cuisine (*Wazwan*), the present work was carried out to study the detection of cattle and buffalo meat in uncooked mutton *Rista* by mitochondrial DNA (mtDNA) based Multiplex polymerase chain reaction (PCR) method under laboratory conditions. The *Rista* emulsions were prepared from mutton, cattle and buffalo meat in the ratios of 60:20:20, 80:10:10, 90:05:05 and 98:01:01 respectively. The primers used in the Multiplex PCR produced characteristic band pattern for each species by amplifying *cyt b* gene fragments of mtDNA of the target species. The size of the amplified bands of *cyt b* gene fragments was 585, 472 and 124bp for mutton, cattle and buffalo meat respectively. Further, the band intensities progressively decreased for cattle and buffalo meat from mixed meat products with their corresponding decreasing level from 20 to 1%. It is thus concluded that the Multiplex PCR is highly effective in detecting adulteration of uncooked mutton product with cattle and buffalo meat up to 1% level.

Keywords: Meat adulteration, multiplex PCR, mutton, rista

Introduction

The higher demand for meat and meat products accompanied by their escalating cost makes them prone to fraudulent adulteration, substitution and mislabeling. The determination of food authenticity and the detection of adulteration are major issues in the meat industry and are attracting increasing amount of attention. Even from religious and ethical reasons, detection of adulteration is of utmost importance (1). Enzyme-linked immunosorbent assays (2) and protein profiles (3) have also been used for meat species identification. Assays based on polymerase chain reaction (PCR) are the method of choice for identification of meat species (4). Various researchers have reported that species-specific PCR analysis of mitochondrial DNA sequences is commonly used method for

identification of meat species in animal feedstuffs (5-7) and food (8-13). The PCR based assays targeting genomic and mitochondrial DNA are being used for the identification of meat species (14-17). The Using species-specific primer pairs for detection of species origin of meat have also been employed for certification of mammalian and poultry species (15-19).

In the state of Jammu and Kashmir, the people are predominantly non-vegetarian, more so in Kashmir valley where meat and meat products are consumed since ages as exemplified by the world famous Kashmiri cuisine, *Wazwan*, with *Rista* being one of the predominant dishes. The fraudulent adulteration of superior quality meat with inferior quality meat is a practice that has been observed all over the world (20).

In view of such likely problem in indigenous meat products of Kashmiri cuisine (*Wazwan*), the present work was carried out to study the detection of cattle and buffalo meat in uncooked mutton *Rista* by mitochondrial DNA (mtDNA) based Multiplex polymerase chain reaction (PCR) method under laboratory conditions.

Materials and Methods

Material and Preparation of Rista: Meat (mutton, cattle and buffalo meat) from leg portion of the respective dressed carcass as well as the respective visceral fat were procured from local market. The product was prepared according to the standardized processing schedule and recipe of Samoon (21) with slight modifications. The basic formulation for *Rista*, prepared under various controls, viz. mutton (CM), cattle (CB) and buffalo (CC) as well as treatments, viz. admixture of three meats respectively in percentages of 60:20:20 (T_1), 80:10:10 (T_2), 90:5:5 (T_3) and 98:1:1 (T_4) is presented in Table 1. In total three experimental trials were conducted under this study.

Preparation of Uncooked Rista Emulsion: The deboned lean meat from each species (sheep, cattle and buffalo) separately was cut into chunks of size 2-2.5 square inch size. The meat in chunks of each species so obtained was subjected to comminution by traditional manual pounding with a wooden hammer called on a flat and smooth round stone called "*Maz-Kaene*" (*Maz* = Meat, *Kaene* = Stone) until it attained a course ground state. All visible connective tissue, tendons, ligaments and blood vessels were separated manually during mincing. Pounding was continued until a fine pasty consistency of minced meat was obtained. In accordance with the formulation (Table 1), weighed portions of minced meat from each species were taken for the desired meat component for control and treatments (admixtures) after thorough mixing. Fresh fat from each species was also finely minced separately and portioned in a similar manner to that of the minced meat. The minced meat and fat component of each control/ treatment was then mixed together and pounded during which common salt and large cardamom seeds were added with periodical sprinkling of required amount of chilled water. Pounding was continued until proper dispersion of lean and fat was obtained and the resultant emulsion exhibited a characteristic cohesiveness, binding and fluidy consistency, traditionally called as "*Macchwor*".

Sampling of Uncooked Products: Random samples of about 50g each were taken from the uncooked emulsion for *Rista* under all the three pure meat controls and their four admixture treatments. The samples so obtained were packaged in properly labeled LDPE bags and frozen stored at -20 °C. For DNA extraction, aliquots were taken from these samples.

Extraction of Mitochondrial DNA: The chemicals utilized for mtDNA extraction from the test samples were Lysis buffer, *Proteinase-K,* TE Buffer, Phenol (Tris saturated, pH 8.0), 10% Sodium DodecyleSulphate (SDS), Chloroform, Isoamyl alcohol, Isopropyl alcohol, Ethanol, 3M Sodium acetate (pH 5.5). The mtDNA from test samples was extracted as per the standard protocol (22) with some modifications. About 300 mg aliquot of the frozen test sample was cut into small pieces with a sterile scalpel and transferred

to autoclaved porcelain mortar. The sample pieces were ground thoroughly by pestle with additions of liquid nitrogen. The test sample homogenate was transferred into a sterile 15 ml tube and liquid nitrogen was allowed to evaporate. Lysis buffer - ST (0.5ml) was added to the tube along with 5ìl *Proteinase K* and 10% SDS (100ìl) to make final concentration of the latter to 2%. The homogenate was incubated for 12-16 hours (overnight) at 55°C. At the end of incubation, the lysate was transferred to an autoclaved 15 ml centrifuge tube and equal volume 0.5 ml of Tris saturated phenol (pH-8.0) was added and mixed gently for 10 min. The lysate was then centrifuged at 10,000 rpm and 15°C for 10 min. The supernatant was transferred into a 2 ml centrifuge tube and half the volume of Tris saturated phenol:chloroform:Isoamyl alcohol (25:24:1) was added and mixed gently for 10 min. It was centrifuged at 10.000 rpm and 15°C for 10 min. The supernatant was transferred into 2 ml centrifuge tube and equal volume of chloroform: Isoamylalcohol (24:1) was added and mixed gently for 10 min and centrifuged at 10,000 rpm and 15°C for 10 min. The supernatant was collected into a 2ml centrifuge tube followed by the addition of 1/10th volume of 3M sodium acetate (pH 5.5) and equal volumes of isopropyl alcohol. The tubes were slowly swirled to precipitate the DNA which was then washed thrice with 70% ethanol and air dried and then dissolved in 200 ìl volume autoclaved triple distilled water using properly marked 2 ml tubes. The DNA samples (stock solution) were stored at-20 °C until further use. Quality, purity and concentration of the extracted DNAs were checked by agarose gel electrophoresis and spectrophotometry.

Primers used for PCR: Various primers used in the present study were as per (23) and are listed in Table 2. The primers were procured from M/s RFCL Ltd., New Delhi and were supplied in freeze-dried form and were stored at -20 °C until their usage.

Multiplex PCR Analysis: The Multiplex PCR was carried out in a thermocycler using 0.2 ml thin wall PCR tubes. The 25ìl PCR reaction volume comprised of 1.6 ìl $MgCl_2$ (1.5 mM), 0.5 ìl M dNTP (0.2 mM), 4.5 ìl of common reverse primer and 1 µl of each forward primer of sheep, cattle and buffalo (0.015 mM), 2 ìl of template DNA (50 ng), 2.5 µl of 10X PCR buffer, 0.06 ìl *TaqDNA polymerase* (0.3 Units) and remaining volumes (10.84 µl) of autoclaved triple distilled water. The optimized PCR protocol comprised of initial denaturation for 3 min at 94°C, followed by 34 cycles of denaturation for 30s at 94°C, annealing at 60°C for 45s, extension at 72°C for 45s and a final extension at 72°C for 10 min. The PCR products were electrophoresed at 85 V for 2 hours in 2% agarose gels after the wells were charged with 5ìl of DNA preparations mixed with 1ìl of 6X gel loading buffer dye and viewed under UV trans-illuminator gel documentation after staining with Ethidium bromide. The sizes of PCR products were determined in relation to a 100 bp DNA ladder.

Results and Discussion

The OD ratio of the extracted DNA from uncooked *Rista* was highest for cattle (1.88) and lowest for buffalo (1.70) with the values of rest of the test samples falling in between the two (Table 3). The data on concentration of extracted DNA from uncooked *Rista* indicated that the values (ìg/ml) ranged from a highest of 1975.00 for T_2 to the lowest of 1725.00 for cattle and T_4. The data of the above referred to parameters indicated that, on an overall basis, the quality of DNA extracted from various samples of the study was good.

The Multiplex PCR using mitochondrial *cyt b* gene specific primers successfully amplified the *cyt b* gene fragments of sheep, cattle and buffalo in each sample of uncooked *Rista* prepared from mixed meats (Fig. 1). The Multiplex PCR amplified fragments exhibited characteristic band pattern that was as expected for the meats of contributing species. The amplified bands of *cyt b* gene were of the sizes of 124bp, 472bp and 585bp for buffalo, cattle and sheep, respectively. The bands of the PCR products of amplified *cyt b* gene fragments of

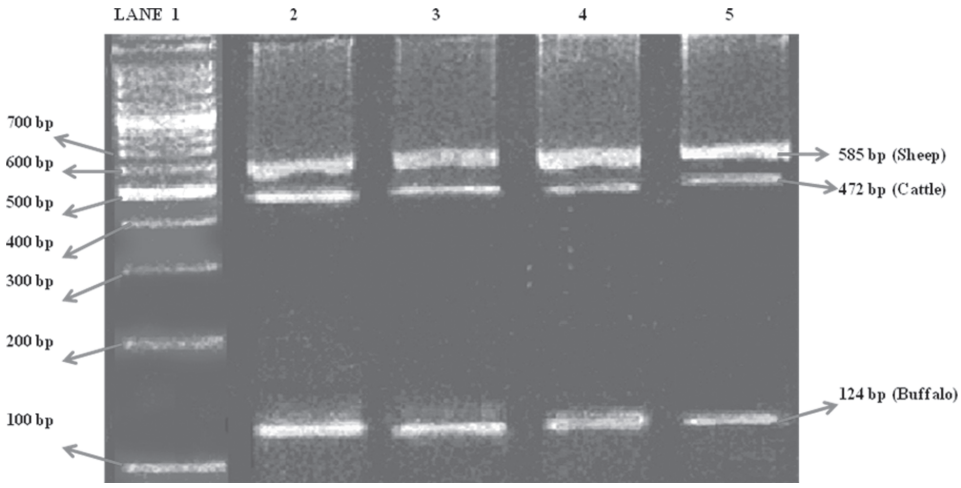

Fig 1. Optimized cyt b Gene Fragment Amplification of Sheep, Cattle and Buffalo from Uncooked Ristaemulsion Prepared from mixed Meats
Lane 1: DNA ladder of 100bp Lane 2-5: Mixed meat (mutton:cattle:buffalo meat in the ratio of 60:20:20, 80:10:10, 90:05:05 and 98:01:01 respectively)

sheep, cattle and buffalo species from uncooked *Rista* prepared from various ratios of mixed meats showing three bands representing the respective three species are presented in Lane 2, 3, 4 and 5. The bands representing mutton of the adulterated emulsion samples were also of good intensities. However, the band intensities for the cattle and buffalo *cyt b* gene fragments showed progressive decline with the reduction of their meat proportion from 20% to 1% in the emulsion. Although, bands representing the adulterant meat (cattle and buffalo meat) showed decline in band intensities with the reduction of adulteration level, but all these bands were visible. Moreover, it was possible to detect cattle and buffalo meat in the mutton Kebab emulsion up to 1% adulteration level. Further, the processing involved in the preparation of *Rista* had no significant effect on meat speciation even up to 1% level.

The results of the present study are in accordance with the reports of earlier workers. Meyer *et al.* (18) has reported the detection of meat species in adulterated mixed meats by tracing 0.5% pork in meat mixture using the duplex PCR technique. Likewise, detection of 1% chicken in mutton using PCR-RFLP (24), 1% pork in cattle labeled meat (11) and 1% chicken meat in meat products by PCR assay (19) has been reported. 0.005% pork in cattle and 1% pork in duck patties by PCR in heated and unheated meats, sausages, canned food, and cured products have been traced (4). Moreover, Calvo *et al.* (25) detected 0.01% uncooked cattle in pork in uncooked emulsion mixture and 1% cattle in cooked meat mixtures by PCR. Similarly, detection of 1% of cattle, sheep meat, goat meat and pork in uncooked and heat treated meat mixtures by PCR assay has been reported (26).

The results of the present study proved the efficiency of mtDNA based test in detecting the meat adulteration even at lower levels, thus making the PCR as a reliable method in the identification of meat species. Among different approaches of PCR, Multiplex PCR is more beneficial for meat speciation because it is highly repeatable, time saving and affordable than the other approaches (27). Among the DNA based techniques, the mtDNA techniques are more advantageous because of the presence of

Table 1. Proportions of meat and fat used in the formulation for *Rista*

Percent Ingredients	Treatments			
	T_1	T_2	T_3	T_4
Mutton	48 (60)	64 (80)	72 (90)	78.40 (98)
Cattle	16 (20)	8 (10)	4 (5.0)	0.80 (1.0)
Buffalo meat	16 (20)	8 (10)	4 (5.0)	0.80 (1.0)
Mutton fat	12 (60)	16 (80)	18 (90)	19.60 (98)
Cattlefat	4 (20)	2 (10)	1 (5.0)	0.20 (1.0)
Buffalo meat fat	4 (20)	2 (10)	1 (5.0)	0.20 (1.0)
Total	100	100	100	100

*T_1, T_2, T_3 and T_4 indicate mixed meat *Rista* with mutton, cattleand buffalo meat as 60:20:20, 80:10:10, 90:05:05 and 98:01:01 respectively.

Table 2. Primers Used in Multiplex PCR

Name	Primer Type	Sequences (5'- 3')	Size (bp)
Common	Reverse	TGTCCTCCAATTCATGTGAGTGT	-
Buffalo	Forward	TCCTCATTCTCATGCCCCTG	124
Cattle	Forward	TCCTTCCATTTATCATCATAGCAA	472
Sheep	Forward	TACCAACCTCCTTTCAGCAATT	585

Table 3. Quality of Harvested DNA from Uncooked *Rista*

Source of DNA Sample	OD_{260}nm	OD_{280}nm	OD_{260}nm / OD_{280}nm	Concentration (ig/ml)	Quality of DNA
Mutton	0.351	0.204	1.72	1755.00	Good
Cattle	0.345	0.183	1.88	1725.00	Good
Buffalo	0.370	0.217	1.70	1850.00	Good
T_1	0.377	0.204	1.84	1885.00	Good
T_2	0.395	0.212	1.86	1975.00	Good
T_3	0.388	0.210	1.84	1940.00	Good
T_4	0.345	0.191	1.80	1725.00	Good

T_1: Mutton:cattle:buffalo in the ratio of 60:20:20 T_2: Mutton:cattle:buffalo in the ratio of 80:10:10
T_3: Mutton:cattle:buffalo in the ratio of 90:5:5 T_4: Mutton:cattle:buffalo in the ratio of 98:1:1.

thousands of copies of mtDNA per cell that ensures a sufficiently large quantity of PCR product even in case of samples undergoing intense DNA fragmentation (28). Further, the chances of mtDNA degradation under different meat processing conditions are lesser, thus making it ideal for processed meat species identification (11).

Conclusion

Therefore, in conclusion the mtDNA based multiplex PCR procedure has the ability to detect

up to as low as 1% level of meat adulteration in uncooked mutton rista, thus making it a method of choice in identification of meat species in adulterated meat.

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Repercussions of Thyroid Profile Hormones Triggered by Oxidative Stress in Infertile Females among the Allahabad-India

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Abstract:

This study has been designed to investigate the impact of oxidative stress on the thyroid hormones profile in the fertile and infertile females of the child bearing age. To achieve this aim 500 married female were included for this study and were divided in two groups: group I: 250 fertile married females and group II : 250 infertile married females falling between the reproductive age 25-35 yrs and having no history of any metabolic disorder. Melondialdehyde (MDA) is considered as oxidative stress marker in the present study. Thyroid profile tests (total $T_{3,}$ total T_4 and TSH) were measured in both the groups. Results indicates from this study that level of MDA showed a significant ($p > 0.0001$) increase in the infertile group in comparison to the fertile group. On the other hand significant ($p>0.0001$) increase in the total $T_{3,}$ total T_4 and TSH in the infertile group when compared to the fertile group. In the correlation study MDA had a significant positive correlation with the T3 ($r= 0.2421, p= 0.00011$) as well as with T4 ($r=0.1463, p=0.020663$).Similarly a significant positive correlation was also found in between the MDA and the TSH ($r=0.020663, p=<0.00001$).

It was concluded that high value of melondialdehyde could be used as a marker for alteration in thyroid profile which is responsible for many complications including fertility rate in females.

Key Words: Infertility, Oxidative stress, Melondialdehyde, Thyroid.

Introduction

Oxidative stress is a condition in which there is an imbalance between the productions of ROS and the ability of the biological system to detoxify the reactive intermediates or easily repair the resulting damage (1). Increase in the level of Reactive oxygen species and decrease in the antioxidant scavenging enzyme leads to the condition of oxidative stress (2). All forms of life maintain a reducing environment within their cells. Normal cellular aerobic metabolism also results in the generation of ROS. Minimal levels of ROS act through signaling pathways, which is necessary for the normal physiological functions in the female reproductive tract (3). The normal cellular metabolism also produce free radicals and are also important to some extent for contribute to normal physiological functions such as cellular differentiation, cell signaling, second messenger pathways, bactericidal activities, and apoptosis as well as adverse effects such as lipid peroxidation, protein oxidation, and DNA damage. Oxidative stress can cause tissue injury or even cell death which can occur essentially by two mechanism, necrosis and apoptosis (4). Oxidative stress also effects the human fertilization and can induce apoptosis which can further result in failure in implantation, fragmentation of the embryo or can cause abortions. Many of the unsuccessful reproductive performance such as infertility, miscarriage and preeclampsia are found to be related to adverse health effects of oxidative stress (5). Successful pregnancy is a combination of a long list of

complex biological steps like, ovulation, production of competent sperm and oocyte in the reproductive tract, proper fertilization, successful transportation of the conceptus to the uterus and implantation of the embryo etc. Disruption in one or more of these complex biological steps can lead to infertility (6). It is proposed that oxidative stress (OS) precipitates the range of pathologies that currently are thought to afflict the reproductive function of female body (7). Study conducted on rats by introducing experimentatal hyperthyroidism was accompanied with increased oxidative stress and with the consumption of antioxidant enzyme in induced oxidative aggression (8). Melondialdehyde (MDA) is the one of the final products of polyunsaturated fatty acids peroxidation in cells. An increase in free radical is causing overproduction of MDA and is commonly treat as a marker for oxidative stress (9).

Material and Methods

The present study were carried out in faculty of Health Sciences, SHIATS and the experimental protocol was approved by the Institutional Ethical Committee in the meeting held on 3rd October 2011, Reg No-2011/A/010. The blood sample of infertile and fertile married females having child bearing age (25-35 yrs) without any metabolic disorder (known from the history of the patient) were collected from different gynecologist clinical hospitals and infertility centers of Allahabad city. The screening of the sample were done on the basis of history given by the females with their written concern as well as performing blood analysis for MDA, and thyroid profile.5ml of venous blood sample were collected from each selected fertile and non fertile married females in Allahabad and were divided in two groups Group I consist of 250 fertile married females having children and Group II includes 250 infertile married females who doesn't have children.

The level of Melondialdehyde was determined by procedure described by Satoh (10**)**) total $T_{3,}$ total T_4 and TSH was measured by Chemiluminescent microparticle immuno assay method (11). Data were analyzed for significance level by Graph Pad online software for T test analysis. This was done by online using www.graphpad.com/quickcals/.

Results

During this study it was found that there was a significant difference in the plasma MDA mean values levels of the two groups, with increase in the group II (3.00±0.295 nmol/ml) as compared to group I (0.85±0.0603 nmol/ml). The results showed significant increment in the MDA value of infertile group which strongly indicates the lipid peroxidation and denotes presence of oxidative stress in the infertile females. Lipid peroxidation measured as MDA content is considered to be indicator of oxidative damage from stress (12)

Results in table 3.2 was reveals that there is a significant difference in the thyroid hormone mean values of the two groups with increase in the group II. The results showed significant increment in the total $T_{3,}$ total T_4 and TSH value of group II when compare to group I. Significantly increased free T_4 values were also observed in the study done (13**)** on rats by inducing experimental hyperthyroidism. Their results suggested that experimental hyperthyroidism (caused by inducing L thyroxin 10µg/animal/day) is accompanied with increased oxidative stress and with the consumption of antioxidant enzyme in induced oxidative aggression.

Correlation Between MDA and Thyroid Hormones in Infertile Females: Table 3.3 representing correlative study among MDA and the Thyroid hormones i.e. T3, T4, TSH. The results indicates a significant positive relation with the thyroid hormones.MDA had a significant positive correlation with the T3 (r= 0.2421,p= 0.00011) as well as with T4 (r=0.1463, p=0.020663).Similarly a significant positive correlation was also found in between the MDA and the TSH (r=0.020663,p=<0.00001).

Discussion

The results show significant increment in the MDA value of infertile group which strongly indicates the lipid peroxidation and denotes

presence of oxidative stress in infertile women. As Melondialdehyde is known to be an oxidative stress marker and is only formed in the lipid peroxidation. The finding of present study was in agreement with the earlier reports or studies (14, 15). The results shows a significant positive correlation of MDA with thyroid hormones.Thus it is indicated that increase in the MDA level causes increase in the level of thyroid hormones which means the condition of hyperthyroidism. In this state of over activity of thyroid gland the female ovulation become irregular or even complete lack of menstrual cycle and thus effect the fertility. The major source of energy production in the body is oxidation which predominantly in mitochondria (16) as well as thyroid hormone also targets mitochondria. There is constant production of hydrogen peroxide during the thyroid hormone synthesis, which is absolutely indispensable for iodine oxidation in presence of thyroid peroxidase. The synthesis of thyroid hormone depends on the hydrogen peroxide, which works as a donor of oxidative equivalents for thyroperoxidase (17). The toxic effect of hydrogen peroxide its synthesis must always remain in equilibrium with the hormonal synthesis. Thyrocytes contains various enzymatic systems ,such as catalase ,superoxide dismutase, glutathionine peroxidase that contribute to limit their limit cellular injuries when hydrogen peroxide or other are produced in excess (18,19,20). The most important factor involved in the regulation of basal metabolic state and the oxidative metabolism is thyroid hormone (21) .Thyroid hormones play a very essential role in the metabolic activities of the body as they can cause many changes in the number and activity of mitochondrial respiratory chain enzyme, which may result in the increased generation of ROS (22,23).

Many experimental studies suggest that hyperthyroidism is associated with a general increase in tissue oxidative stress. Some studies suggest the thyroid hormone, especially thyroid stimulating hormone (TSH), plays a role in infertility (24). Pregnancy as well as various aspects reproduction is disrupted by thyroid dysfunction.

Many studies highlight role of thyroid hormones as disturbance in their levels in the clinical conditions such as hyperthyroidism or hypothyroidism causes disturbance in menstrual cycle causing anovulatory cycles and increases morbidity in pregnancy (25, 26, and 27). It is known that thyroid hormones have major effects on the female reproductive system (28). They are critical for growth, development and differentiation. *In vitro* and *in vivo* data indicate that anovulations of estrogen and thyroid hormone levels can alter each other's functions. One possible mechanism for interaction may be that thyroid and estrogen receptors, having structural similarities, bind to an identical half-site, AGGTCA, of their cognate hormone response elements and thereby can compete with each other at this level (29).

.**Conclusion**

With the results observed in this study it may be concluded that oxidative stress, adversely affect the female reproduction and may also lead to infertility. As ROS are naturally made up by the human body and is a part of normal healthy metabolic activity but increased amount of ROS burdens immensely the fertility rate of the females. Thus to counter the ROS, the protective attempt i.e. antioxidants supplementation in the diet may be very beneficial for the body. The a positive correlation indicates that a change in the value of one variable will predict a change in the same direction in the second variable i.e increase in one variable may also cause increase in the second variable whereas the negative correlation result indicates that the change in the value of one variable predicts a change in the opposite direction in the second variable i.e increase in one variable may cause decrease in the other variable and vice versa. Thus the result indicates that high value of MDA level may act as trigger for thyroid hormones which mean the condition of hyperthyroidism or over active thyroid gland. In this state of over

Table 3.1. Serum MDA level in Fertile female and Infertile female.

S.No	Parameters	Group I	Group II	t-test
1	MDA	.85±.0603	3.00±0.295	126.111*

Normal Range: MDA (nmol/ml):0.5-2.0

Table 3.2. Thyroid hormone profile in fertile and infertile females.

S.No	Parameters	Group I	Group II	P.Value Significance	t-Test
1.	Total T_3 (Triiodothyronine) (ng/mL)	91±1.107 .	2.02±1.293 significant (>0.0001)	Statistically	10.31
2.	Total T_4 (Thyroxine) (µg/dl)	6.48±1.587	11.66±1.742	Statistically significant (>0.0001)	34.75
3.	TSH (Thyroid stimulating **hormone)** (µIU/ml)	1.48±.603	4.47±1.069	Statistically significant (>0.0001)	**38.51**

T_3 (Triiodothyronine) (ng/mL): 0.58 - 1.59 Total T_4 (Thyroxine) (µg/dl): 4.50-12.60
TSH (Thyroid stimulating hormone) (µIU/ml):0.35-5.5

Table 3.3. Correlation factors of serum Melondialdehyde with the thyroid function test i.e. T_3, T_4, TSH (thyroid stimulating hormone) in infertile women

MDA Verses	Parameters	r value	p. value	Significance level
Thyroid	**T_3**	**0.2421**	0.00011	Significant at p<0.05
hormones.	**T_4**	**0.1463**	0.020663	Significant at p<0.05
	TSH	**0.020663**	<0.00001	Significant at p<0.05

activity of thyroid gland it causes the overt activity of metabolism. It also effects the female ovulation as it causes irregular or even complete lack of menstrual cycle and thus affecting the fertility.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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In vitro Propagation for Rapid and Efficient Regeneration of *Cardiospermum halicacabum* L. – An important Medicinal Plant

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Abstract

Cardiospermum halicacabum, a medicinial plant rich in β-sitosterol, D-glucoside, saponin and quebrachitol. Direct regeneration was achieved only from the nodal explants which showed 100 per cent sprouting on MS medium supplemented with BAP (0.5 ppm) + NAA (1.0 ppm) in 12 days, while MS medium supplemented with BAP (0.5 ppm) + IBA (1, 2 or 3 ppm) showed early but decreased sprouting. The half strength MS basal medium without any growth regulator proved to be best medium for root induction for excised shoots. The *in vitro* raised plantlets were transplanted to mixture of sterile dune sand and FYM (3:1) with moderate (80 %) success.

Key words: *Cardiospermum halicacabum*, nodal explants, axillary buds, growth regulators.

Abbreviations : MS, Murashige and Skoog; 2, 4-D, 2, 4-dichlorophenoxy acetic acid; IAA, indole acetic acid; IBA, α-indole 3-butyric acid; FYM, Farm Yard Manure; NAA, α-naphthalene acetic acid, BAP, 6-benzyl amino purine; Kn, kinetin.

Introduction

The World Health Organization (WHO) has enlisted over 21,000 plants having medicinal and therapeutic values. Out of this, more than 2000 plant species are used in traditional medicines of India, as described in texts *Charak Samhita* and *Sushruta Samhita*. Globally, about 3.5 billion people rely on these traditional medicines for their health care needs (2). *Cardiospermum halicacabum* L. (Ballon Vine) is an annual or perennial climber belonging to the family Sapindaceae. It is commonly found throughout India up to an elevation of 1200 m. It is recognized as a medicinal plant of repute in Ayurvedic and Homoeopathic system of medicine (12). It has diuretic, stomachic, antispasmodic and rubefacient properties and is used to treat rheumatism, nervous disorders and dropsy (5). Its leaves contain β-sitosterol and D-glucoside, saponin and quebrachitol as pharmaceutically important compounds. Besides, it also contains many important alkaloids like stigmasterol, proanthocynidia and apigenin (1). The plant extract shows *vasodepressant* activity and has blood purifying properties. The juice of leaves is given to reduce obesity. The decoction of roots is useful in curing gonorrhea, nervous diseases, haemorrhoids and erysipelas. The powder of seeds is useful for the treatment of cancer (1).

Micropropagation plays an important role in the sustainable utilization of plants and is

especially valuable for the commercial cultivation of such plants. Often these plants are accessed from the wild areas and forests that generally results in excessive harvesting. In many cases, the entire plant is cut off for use and this practice may threaten the existence of overexploited species. In order to prevent overexploitation as well as to promote conservation of such medicinal species through cultivation, availability of elite planting material is a prerequisite. Conventionally, *Cardiospermum halicacabum* is multiplied through seeds, which have low viability, poor germination and show insufficient and delayed rooting in seedlings. This has necessitated the development and standardization of alternative protocols for the rapid multiplication of this valuable medicinal plant (5). Plant propagation through sexual (seed) means is not only a slow process but the seedling population also often exhibits considerable variability for important traits. In medicinal plants, this variability may result in poor levels and inferior quality of different active ingredients in the harvested tissues. Plant tissue culture techniques provide an attractive option for the rapid and efficient multiplication of different plants (8, 9). In *Cardiospermum halicacabum*, only a few reports are available on plant regeneration though callus culture but practically no information is available regarding direct organogenesis (5). In this study we report protocol for an efficient *in vitro* multiplication of *Cardiospermum halicacabum*.

Materials and Methods

Plant Material: The nodal explants obtained from the field grown plants of *Cardiospermum halicacabum were* thoroughly washed in the running tap water followed by washing with 1.0 % teepol solution for 10 min by vigorous stirring. Subsequent operation was performed aseptically under a laminar air flow cabinet. The explants were surface sterilized with 0.1% mercuric chloride (2 min) followed by 70% ethanol (1 min) and thereafter washed 4-5 times with sterile distilled water to remove the traces of mercuric chloride. The surface sterilized explants were inoculated on MS (10) basal medium alone and with various concentrations of different auxins (2, 4-D, IBA, NAA) and cytokinins (BAP, Kn) - either alone or in different combinations.

Experimental Conditions: The 30-40 ml of MS medium containing 3% sucrose and 0.8% agar along with growth regulator(s) was poured into 150 ml flasks. The pH of the medium was adjusted to 5.8 with 1N sodium hydroxide (NaOH) and/or 1N hydrochloric acid (HCl), prior to the addition of agar and the medium was subsequently autoclaved at 121ºC (1.2 Kg cm^{-2} pressure) for 15 min. All the chemicals used were of analytical grade. All the cultures were incubated at 26 ± 2 ºC under white fluorescent light with a photoperiod intensity of 2000 lux (16 h of light and 8 h of darkness).

All experiments were repeated twice, using 10 replicates (flasks) each containing three explants. The data were analyzed statistically using completely randomized design and the significance was tested at 5% level of critical difference using OPSTAT software (CCS HAU, Hisar).

Results and Discussion

Shoot Regeneration: Direct regeneration in *Cardiospermum halicacabum* cultures was achieved only from the nodal explants. On MS medium devoid of growth regulators, 66% axillary bud sprouting took place in 21 days after inoculation (Table 1). On MS medium supplemented with 2, 4-D (0.5, 1, 1.5 and 2 ppm), no sprouting was observed even after 4 weeks of inoculation. In contrast, supplementation with Kn (1.0, 1.5 and 2.0 ppm) or use of BAP in higher concentration (4 ppm), resulted in 100% sprouting between 13-17 days after inoculation (Table 1; Figs 1a, 1b). Shoot proliferation directly from the nodal explant could be attributed to the presence of pre-existing axillary primordial, which proliferated into shoots under adequate concentration of cytokinin supplied in the nutrient medium.

Synergistic effects of plant growth regulators have influenced the cultural response in shoot proliferation and elongation. However, the number and percentage of sprouting of axillary buds varies with the species as well as with hormone concentration. Similar results have been reported by Singh (16) in *Chlorophytum borivilianum,* Sharma *et al*. (15) in *Vitex negundo* and Kumar *et al*. (9) in *Dioscorea alata.*

When BAP (0.5 and 1 ppm) along with IBA (1, 2 and 3 ppm) was used in the medium, 75-82% sprouting was obtained between 7-11 days after inoculation. It was also noted that increase in concentration of IBA in the medium resulted in delayed but significantly higher sprouting, i.e. from 75% in IBA (1 ppm) to 82% in IBA (3 ppm). In MS medium supplemented with BAP (1 ppm) and NAA (1 ppm), shoots were formed between 7-12 days of inoculation with 100% sprouting. Use of higher concentrations of NAA (above 1 ppm) along with BAP (1 ppm), resulted in no sprouting. It was also observed that only one plantlet was normally formed from each axillary bud (Table 1; Fig 1c). It appeared that supplementation of nutrient medium with adequate concentrations of cytokinin and trace amounts of auxins overcame the effects of apical dominance and thus enhanced the proliferation of axillary buds (7, 9). These results are in accordance with earlier reports on Mose rose (14) and *Dioscorea alata* (9).

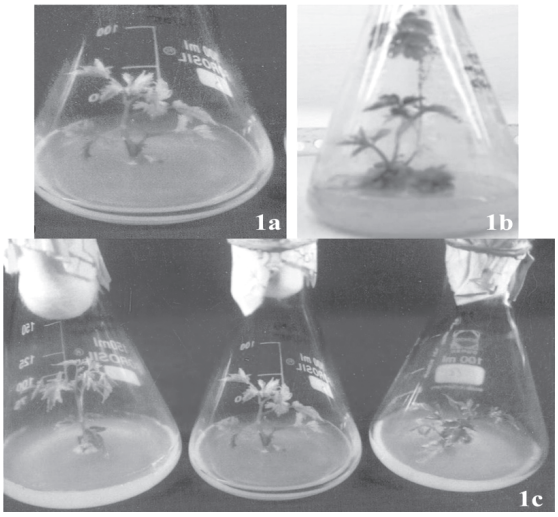

Fig. 1. Direct shoot regeneration from nodal explants on MS medium supplemented with 4.0 ppm BAP (Figure 1a); 1.0 ppm Kn (Figure 1b); and 1.0 ppm BAP + 1.0 ppm NAA (Figure 1c).

Rooting of Shoots: The regenerated shoots (3-5 cm in length) derived from the axillary buds of nodal explants (direct regeneration) were transferred on the MS basal medium supplemented with different strengths of mineral salts (¼MS, ½MS and full strength MS basal medium) for rooting. The rooting was in range of 67-100% under media with different strength of mineral salts and the maximum number of roots (8-9; 3.0-4.0 cm in length) were formed at ½MS strength basal medium (Table 2; Fig 2a). Similar results have earlier been reported in *Acacia catechu* (17), *Centarium erythraea* (11) and *Hedychium spicatum* (6), where ½ strength MS medium was also used for rooting.

Acclimatization of in Vitro Raised Plantlets: The plantlets developed were taken out of the medium after sufficient development of roots and were subsequently transferred to small pots containing sterilized sand and FYM (3:1). The pots, covered with polythene bags to maintain the high humidity and kept in culture room at 26 ± 2ºC, were irrigated with ½ strength MS salt solution from time to time. After about 2 weeks, the potted plants were taken out from the culture room and polythene bags were removed for 3-4 h daily to expose the plant to the natural humidity conditions for acclimatization (Fig 2b). These acclimatized plants were then transferred to the bigger pots under field conditions. During this process of hardening some plants could not withstand the transplanting shock and died after few weeks. After about 4 weeks, these plants were subsequently shifted to the pots containing soil and sand mixture in the Botanical Garden under natural light, temperature and humidity

Table 1. *In vitro* response of nodal explants of *C. halicacabum* on MS medium supplemented with auxins and cytokinins (in different combinations).

Medium + growth regulator (ppm)	Explant callusing (%)	Days required for callus induction/ sprouting of axillary buds*	Visual growth of callus after 4 weeks	Colour and texture of callus (sprouting of axillary bud)
Control	66	36/21	+	Green, fragile (sprouting)
2,4-D (0.5)	87	11/—	+	Light green, compact
(1.0)	100	11/—	+	Light green, compact
(1.5)	100	11/—	+	Light green, compact
(2.0)	100	11/—		Light green, compact
Kn (0.5)	75	11/—	+	Light green, compact
(1.0)	100	11/15	+	Light green, compact
(1.5)	100	11/15	+	(sprouting)
(2.0)	100	11/13	+	Light green, compact (sprouting)
				Light green, compact (sprouting)
BAP (1.0)	75	30/—	+	Green, compact
(2.0)	80	26/—	+	Green, compact
(3.0)	82	25/—	+	Green, compact
(4.0)	100	22/17	±	Green, compact (sprouting)
IBA (1.0) +BAP (0.5)	75	21/7	+	Green, compact (sprouting)
IBA (2.0) +BAP (0.5)	80	21/9	+	Green, compact (sprouting)
IBA (3.0) +BAP (0.5)	82	21/11	+	Green, compact (sprouting)
NAA (1.0) +BAP (1.0)	100	36/12	+	Green, compact (sprouting)
NAA (1.5) + BAP (1.0)	100	36/—	+	Green, compact
NAA (2.0) + BAP (1.0)	97	36/—	+	Green, compact

Data presented are means of 5 replicates
* — No sprouting, + Poor callus, ++ Moderate callus.

Table 2. Formation of roots from excised shoots regenerated *in vitro* from axillary bud break from nodal segments on various strengths of MS medium (observation recorded upto 4-weeks after inoculation).

Medium strengths	No. of days required for root induction	% root induction	No. of root per shoot/ (length in cm)
Full MS basal medium	6	67	2-3/(1.5-2.0)
½ MS basal medium	4	100	8-9/(3.0-4.0)
¼ MS basal medium	4	100	4-5/(2.5-3.5)

Data presented is mean of three replicates

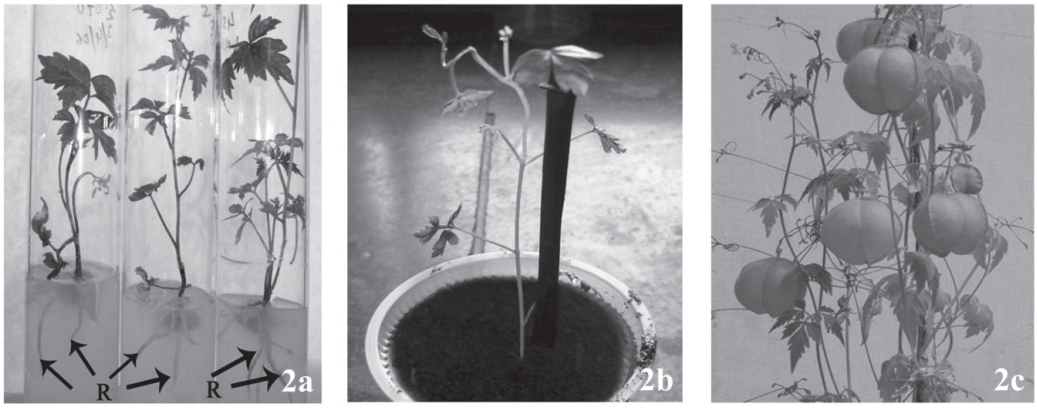

Fig. 2 Root induction from *in vitro* regenerated shoots on half strength MS medium in *C. halicacabum* (Figure 2a); 2 months old plant in pot before transferring to the field conditions (Figure 2b); and fully matured plants of *Cardiospermum halicacabum* (Figure 2c).

conditions (Fig 2c). Similar acclimatization procedure for successful establishment of *in vitro* regenerated plants has been demonstrated by Purohit *et al.* (13), Chukwujekwu *et al.* (4) and Singh (16).

Conclusion

An efficient and repeatable method for was developed *in vitro* micropropagation of *C. halicacabum*. Regeneration was found to be dependent on synergistic effect of cytokinins and auxin in the MS medium and the best result of multiple plantlets regeneration was obtained from nodal explants when BAP (1.0 ppm) along with NAA (1.0 ppm) supplemented to MS medium. The rooted shoots were hardened for survival after transplantation to mixture of sterile dune sand and FYM (3:1) in pots by exposing them to subsequent humidity conditions. These were then transfer to the soil with moderate (80 %) success. The plant regeneration protocols described here can be used for mass propagation and to provide plant material for improvement programs of the cultivated species. Those based on the development of preexisting meristems and direct organogenesis is particularly useful for *in vitro* conservation.

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Fish Processing Waste: A Promising Source of Type-I Collagen

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Abstract

Collagen is the most abundant protein of the animal kingdom. It has varied application in different fields such as pharmaceutical, biomedical, cosmeceutical, etc. Among the twenty nine different types of collagen identified till date, type I is the most prominent one. Most of the marketed collagen is obtained mainly from the animal source which includes bovine and porcine skin and bones, chicken waste, etc. But due to its high cost and the onset of diseases such as FMD(Foot-and-Mouth Disease), BSE (Bovine spongiform encephalopathy) and TSE, cheaper and safer sources of collagen have been probed for. Thus the role of marine source came in which has been acknowledged as a promising alternative collagen source owing to the advantages such as absence of zoonosis risks, lack of dietary constraint, easy availability and higher yields.

Fish processing waste is a major environmental hazard. It includes both the solid and liquid waste. The solid waste, which includes the remains of fish such as its head, skin, scales, fins, etc, discarded after the step of processing poses the foremost threat to the environment. Presently these offals are made use of as a source of value added products such as proteins, minerals, gelatin, bioactive peptides, fish oils, enzymes, biogas/biodiesel, amino acids, collagen, etc. Use of this waste for collagen production would not only save our environment, but also be the cheapest source of raw material. It is a potential source of collagen which has been proved from the research work carried out utilising the same. Thus, this review article summarises the work carried out to obtain type I collagen from the wastage of different fishes.

Keywords: Type-I Collagen, Fish waste, ASC, PSC, Marine source.

Introduction

Collagen is a structural fibrous protein found abundantly in animals accounting for nearly 30% of the total protein present in their body. It is mainly present in extracellular matrix of various connective tissues such as the skin, cartilage, blood vessel, bone, teeth, ligament, tendon, etc. (1). It plays a crucial role in maintaining the structure of different tissues and also helps in tissue remodeling, adhesion, etc. Collagen is mainly obtained from porcine and bovine source. But due to its high cost, onset of diseases (such as FMD, BSE and TSE) and religious hindrances, cheaper and safer sources of collagen have been probed for. Thus the role of marine organisms came in which has been acknowledged as a promising alternative collagen source owing to their advantages which includes absence of zoonosis risks and religion based dietary constraint, easy availability and higher yields, etc. They are abundantly present in the vertebrates as well as invertebrates. In case of vertebrates,

collagen forms the structural element of skin, bone, cartilage, etc; whereas in the invertebrates, they are present in their body walls, cuticles, etc.

Collagen is a naturally occuring protein. The collagen molecule is made up of three polypeptide chains (á chains). These chains may be identical or two or three of them may be different.Each chain exhibits a left handed helix conformation. The three chains are twisted to form a triple helix which is stabilised by the presence of hydrogen bond. The molecule exhibits a distinct tertiary structure due to its characteristic repetitive sequence of triplet Gly-X-Y with each chain being more than 1000 residues long. Collagen types differ based on the different X and Y ie the amino acids linked to glycine. Mostly, X and Y are the imino acids proline and hydroxyproline.

Presently there are 29 different collagen types available. Fibril forming collagen represents almost 90% of the collagen present in our body. It includes collagen type I,II,III,V, XI, XXIV, AND XXVII (2,3). Among these type I collagen is the most abundant one. Its abundance is an outcome of its extensive occurrence in almost all connective tissues present in the body except hyaline cartilage (4,5). It comprises nearly 90% of the organic bone mass. It is prominently found in the skin, tendons, ligament, cornea, ligature, etc. (6). The triple helix of type I collagen is a heterotrimer with two identical á 1(I)-chains and one á 2(I)-chain. á 2 chain which is hydrophobic in nature, stabilises the collagen (7).

Collagen has diverse application in various fields. It is used mainly in the biomedical, pharmaceutical and medical industry. Its applicability as a biomaterial in drug delivery systems and in tissue engineering is based on the unique characteristics such as high biodegradability, biocompatibility, cell attachment capability, high tensile strength, weak antigenecity, abundance, easily purifiable, etc. (8-13). It is also used in the food, cosmeceutical, film and leather industries etc. (14-16).

Currently type-I collagen is considered as the gold standard in the field of tissue engineering (17) collagen based biomaterials have prominent application especially the collagen scaffold. It is utilised for the study of cell behaviour, as nervous system models, testing of anti-cancer drugs to cultivate ex-vivo organs, as 3-D model for bone diseases, etc. (18-29). Type-I collagen is obtained industrially from bovine achilles tendon. Due to its soaring cost, cheaper sources of collagen are on the look out. Thus came in the role of fish processing waste as a source of collagen. Collagen obtained from the wastage of fishes have been utilized in diverse fields. A few examples of the same obtained from the processing waste are given below:

- The collagen from the swim bladder of marine cat fish (Tachysurus maculatus) has been used in the medical field as a wound healing matrix in the form of collagen-chitosan sheet.
- Lates calcarifer scales have been used as dressing material for wounds in the form of collagen sheet.
- Collagen type I from the outer skin of marine eel fish (Evenchelys macrura) is used as drug delivery system.
- Collagen from swim bladder of Bester sturgeon fish in the form of hydrogel has potential to be used as biomaterial in the field of tissue engineering.
- Collagen based biomaterial using swim bladder matrix of Rohu (Labeo rohita).

Fish Processing Waste

In the current scenario, fish processing waste poses a major threat to our environment by causing pollution. The wastes are both in solid and liquid form (30). The solid waste consists of tail, head, skin, scales, fin , gut, etc. It accounts for nearly 75% of the total weight of fish, (even after filleting) out of which the skin and bones makes up 30%. These offals can be made use of as a source of value added products such as proteins, minerals, gelatin, bioactive peptides, fish oils, enzymes, biogas/biodiesel, amino acids,

collagen,etc (31). The usage of FPW increases the economic value of the fish also and helps to increase the income. In this review we have focused on summarising about type I collagen extracted from these discards, which has not been done so far.

Skin : Fish skin accounts for nearly 6% of the live weight of fish especially in carps. It forms a major portion of the processing waste and is easily obtainable and abundant. It is considered a significant source of highly soluble collagen (32). The main component of skin is type I collagen. Due to the above reasons, majority of the extraction of type I collagen has been carried out using fish skin. Usually fish skin is used as soil fertilizer or animal food supplement. If it is used to obtain collagen, it will definitely increase the demand of the waste.

Collagen type-I has also been extracted from the skin of Striped catfish *(*Pangasianodon hypophthalmus)*,* rock fish (Sebastes schlegeli), marine eel fish (Evenchelys macrura), silver-line grunt, albacore tuna, brown backed toadfish, baltic cod, nile perch (young and adult), cat fish (Tachysurus maculatus),Catla catla, longtailtuna Thunnustonggol, Cirrhinus mrigala ,Alaska pollack, etc. (33-38). Nileperch, Leather jacket and Japanese sea-bass skin have yielded the highest amount of collagen compared to the other fish skins. When comparing the denaturation temperature Marine eel fish and Niletilapia have values close to mammalian range. Collagen type I obtained from the skin of different fishes have been included in table 1.

Bones : Bones are separated after the muscle proteins are removed from the fish frames. It contains nearly 30% of the collagen in all. The minerals present in them are calcium, phosphorous, hydroxyapatite, etc (31). Type I collagen can also be obtained from the bones of Leather jacket (Odonus niger), etc. Ayu has the highest yield of collagen compared to the other fish bone extracts. Collagen type I obtained from the bones of different fishes have been included in table 2.

Scales : Collagen type-I was also obtained from black drum, sheep's head sea bream, red tilapia, skipjack tuna, ayu, yellow sea bream and horse mackerel, pagrus major, Catla catla, Cirrhinus mrigala, lates calcarifer, Pacific saury (Cololabis saira), lizard fish (Saurida spp.) and horse mackerel (Trachurus japonicus) from Japan and Vietnam and grey mullet (Mugil cephalis), flying fish (Cypselurus melanurus) and yellowback seabream (Dentex tumifrons) from Japan, threadfin bream (Nemipterus japonicas), etc. (55-59). Sardine scales yielded the highest amount of collagen. Denaturation temperature (Td) of Grass carp was closest to mammalian value.

Collagen type I obtained from the scales of different fishes have been included in table 3.

Fins : Collagen type-I is extracted from the fins of different fishes such as Catla catla, Cirrhinus mrigala, Japanese seabass, longtailtuna Thunnustonggol, Tilapia, threadfin bream (Nemipterus japonicas) ,etc (63,64). The caudal fin of Japanese seabass was used to extract collagen which yielded higher acid insoluble collagen(36.4% dwb) when compared to ASC(5.2% dwb). The denaturation temperature was in the range 28.0–29.1°C (65,66).

Muscle : Type-I collagen is obtained from the muscle of fishes such as the Atlantic salmon,Amur sturgeon, catfish, carp, etc. (67). Atlantic salmon yielded 23.7% ASC, 70.5% PSC and 5.8% in-soluble collagen (ISC) (68). Amur sturgeon gave ASC 31.56%, PSC 58.49% and SSC(salt-solubilized collagen) 3.02% with a Td of 33°C (70). ASC 97.523 mg/g dwb and PSC 368.360 mg/g dwb was obtained from the Cultured Clarias species (hybrid of Clarias gariepinus×C. macrocephalus), a freshwater catfish (70). The other fishes used were eel, saury, mackerel , chum salmon, carp, etc. The Td of collagen from muscle was comparitively higher than that of skin (71).

Swim bladder : Collagen type-I obtained from the swim bladder of Marine cat fish (Tachysurus maculatus) yielded 35% of collagen in its

Table 1. Type I collagen obtained from the skin of various fishes.

Fish	Yield & type of collagen	Denaturation temperature	Ref
Japanese sea-bass	51.4%	25.0–26.5°C	(39)
Chub mackerel	49.8%	25-28°C	"
Bullhead shark	50.1%	"	"
Nile tilapia (Oreochromis niloticus)	38.84(N) and 20.70% (O) dwb(ASC) 48.21 (N) and 38.27%(O) dwb (PSC)	34.43 (O)and 4.29°C 3(N) (ASC) 34.61 (O) and 34.32°C (N) (PSC)	(40)
Malaysian catfish (hybrid *Clarias sp.gariepinus*)	ASC,PSC :18.11±0.32 and 26.69±0.54% (wwb)	31.5 and 31.0°C	(41)
Unicorn leatherjacket (Aluterus monoceros)	3.39 and 28.33%dwb (ASC & PSC)	30.03°C	(42)
Ocellate puffer fish	10.7% dwb (ASC) 44.7% dwb (PSC)	-	"
Grass carp	46.6% (PSC)	28.4°C	(43)
Blackcarp (Mylopharyngdon piceus)	45.7% dwb(PSC)	25.6°C	(44)
Nileperch (Lates niloticus)	Young fish: 63.1% Adult fish: 58.7% dwb(ASC)	36 °C	(45)
Leather jacket (Odonus niger)	46–50% (ASC) 49–58% (ASC and PSC) 64–71% (PSC)	Skin collagen - 27 to 28 °C bone collagen -31 to 32 °C	(46)
Brown stripe red snapper (Lutjanus vitta)	9%wwb(ASC) 4.7%wwb(PSC)		(47)
Blackpomfret (Parastromateusniger)	13.6% dwb(ASC)	-	(48)
Siganus sutor	12-14 %	-	(14)
Narroe barred	14-17%	-	"
Carcharhinus leucas	14-15%	-	"
Indopacificking mackerel	10-12%	-	"
Bigeye snapper (Priacanthus macracanthus)	6.4% & 1.1% wwb (ASC & PSC)	-	(49)
Rainbow trout (Onchorhynchus mykiss)	9.448% and 1.122% wwb (ASC)	-	(50)
Surf smelt (Hypomesus pretiosus japonicus brevoort)	24% dwb (ASC)	32.5°C	(51)
Barramundi (Lates calcarifer)	Pepsin (PSC) and papain (pASC) aided extraction 43.6% and 43.9%, ASC- 8.1% (dwb)	-	(52)
Double-spotted queenfish (Scomberoides lysan)	ASC, pdc 7.82, 3.92	-	(53)
Malabar grouper (Epinephelus malabaricus)	ASC, pdc 12.5 and 6.49%	-	"
Marine eel fish (Evenchelys macrura)	ASC-80%,PSC-7.1%dwb	38.5°C (ASC)35°C (PSC)	(32)

Where, ASC: Acid Soluble Collagen,PSC:Pepsin Soluble Collagen, N- Noitup method, O: Ogawa method, PDC: Pepsin Digestible Collagen, dwb:dry weight basis, wwb: wet weight basis.

Table 2. Type-I collagen extracted from the bones of different fishes.

Fish	Yield & type of collagen	Denaturation temperature	Reference
Skipjack tuna	42.3%	29.5–30.0°C	(46)
Japanese sea-bass	40.7%	-	‘’
Ayu	53.6%	-	‘’
Yellow sea bream	40.1%	‘’	‘’
Horse mackerel	43.5%	-	‘’
Black drum and sheepshead seabream	PSC	-	(54)
Rainbow trout (Onchorhynchus mykiss)	9.448% and 1.122%(ASC)	-	(50)

Table 3. Type-I collagen obtained from the scales of various fishes.

Fish	Yield & type of collagen	Denaturation temperature	Reference
Sardine	50.9% dwb (PSC)	-	(60)
Red sea bream	37.5% dwb (PSC)	-	“
Japanese sea bass	41%dwb (PSC)	-	“
Carp fish (Cyprinus carpio)	ASC, PSC	ASC: 32.9°c PSC :29.0°c	“
Grass carp(Ctenopharyngodon idellus)	25.64% dwb(PSC)	35-40°c	(61)
Spotted golden goatfish (Parupeneus heptacanthus)	0.46% & 1.2% dwb(ASC & PSC)	-	(62)

lyophilised dry weight basis when PSC method was used. Other fish swim bladder from which collagen is obtained includes Bester sturgeon fish,Rohu (Labeo rohita), Arius parkeri (Gurijuba), yellowfin tuna (Thunnus albacares),bighead carp (Hypophthalmichthys nobilis), Cynoscion acoupa (Pescada Amarela), Cynoscion leiarchus (Pescada Branca), Alaska pollack, etc. (72-76). Yellow fin tuna yielded 1.07% ASC and 12.10% PSC. The biological properties of the collagen obtained from the swim bladder of fishes are to be validated for their application as a polymeric material which is biocompatible in nature and has potential to be used for dressing wounds (in controlled drug delivery systems),as injectables in case of coating cardiovascular prostheses and as a support for cell growth.

Conclusion

Fish processing waste which is discarded after the step of processing poses a threat to the environment. It not only causes water pollution, but also air pollution due to the foul smell they emanate. FPW is presently used for various purposes such as production of gelatin, collagen, enzymes, etc. From the above review, we can conclude that fish waste (particularly its processing waste) is an excellent source of collagen especially type-I. And the use of this economic and abundant waste as a starting material for collagen production would also

contribute for the protection of our environment. The collagen type I has already been used in various fields such as medical (as dressing material for wound healing), pharmaceutical (as drug delivery systems), biomedical (as biomaterial for tissue engineering purposes), etc. to name a few. Many more such applications of type-I collagen are yet to be identified and utilized for the welfare of mankind. Apart from the type-I, other types of collagen have also been obtained from their wastes. Extensive studies have been carried out till date for extracting collagen from fishes and other marine source as well (including vertebrates and invertebrates). If the research on collagen isolation is intensified further, it may reveal even better and more reliable sources of collagen type I with promising yields.

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DNA Barcoding as an Authentication Tool for Food and Agricultural Commodities

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Abstract

Adulteration detection and food authenticity testing are important for value assessment and to ensure consumer satisfaction.New generation DNA based adulteration detection methods such as DNA barcoding is becoming an important tool either as to complement the existing physical,sensory,or biochemical analytical methods or as a stand-alone tool due to its cost effectiveness, high through put,sensitivity and reliability. DNA barcoding is a relatively simple technique that is based on the sequence variation in short nucleotide regions called barcodes that enables species identification and commodity authentication. This review provides an insight into the emerging potential of DNA barcoding for authentication of food and agricultural commodities like seafood, spices, medicinal plants, tea etc.

Keywords: Adulterants, Barcoding loci,DNA markers, Food safety, Value assessment.

Introduction

Food adulteration is an issue of concern having a major social, economic and environmental impact. Adulteration may be defined as mixing or substituting the original material with other spurious, inferior, defective, spoiled, useless parts of the same or different plant or harmful substances, synthetic chemicals which do not conform with the official standards. Adulteration can be in two ways - direct/ intentional adulteration and indirect/unintentional adulteration. Direct/intentional adulteration includes practices of substitution partially or fully with inferior materials owing to their morphological resemblance or chemicals or inert materials in order to attain economical gain. Unintentional adulteration results mainly due to the absence of a proper evaluation method (1), negligence and clerical errors (2) etc.

Adulteration detection and authenticity testing of food and agricultural commodities of plant origin including cereals, legumes, beverages, olive oil, fruit products, spices and traded medicinal plant materials are important for value assessment, to check unfair competition and of all to ensure consumer protection against fraudulent practices commonly observed in unscrupulous trade. Also, deceitful adulteration of these products containing, undeclared constituents may cause intoxication or problems such as allergy in sensitive individuals (3,4, 5).

Detection of adulteration and determination of authenticity and quality of foods and food ingredients, including spices are major challenges for the agricultural and food industry, issues that have become increasingly important in recent years (6). Regulatory agencies, food processors and consumers are all interested in detecting adulterants or authenticating raw materials of food products in order to satisfy food quality and safety requirements (7). With globalisation of trade, the role of standards and conformity assessment are of paramount importance because non-tariff agreements such as Sanitary and Phytosanitary (SPS) and Pre

shipment Inspection (PSI) agreements insist that the product(s) is safe, free from adulterants and has the desired quality.

International organisations like the International Organisation for Standardisation (ISO), American Spice Trade Association (ASTA),US Food and Drug Administration (FDA), The Food Safety and Standards Authority, India (FSSAI) etc. impose strict regulations on the quality of food products, spices and herbs imported and exported. Globalisation of food trade requires the development of integrated approaches, such as traceability of origin, quality and authenticity to ensure food safety and quality (8). In the post-WTO era, importing countries as well as the consumers pay more and more attention to food quality, demanding clearer product traceability as well as the use of detailed and accurate product labels.

Numerous techniques have been developed to counter adulteration owing to the increased consumer awareness of food safety and quality control. Adulteration determination is mainly accomplished by comparing measured analytical data with a proper reference set of historical or control data (9). Three strategies are employed for demonstrating admixture in agricultural commodities: demonstrating the presence of a foreign substance in the commodity, demonstrating that a component is present at a concentration which deviates significantly from its normal level and checking the chemical profile of the sample of which the first strategy is considered as the simplest and efficient (3). Authentication tools utilised vary widely depending on the commodity and processes involved ranging from structural evaluation using physical methods, chemical profiling-based analytical methods and the most advanced biotechnological approaches (10).

Physical methods involved in the authentication are macroscopic and microscopic structural evaluation and other parameters such as solubility, bulk density, texture etc. (3). Analytical methods used for food authenticity testing involve chromatographic techniques like High performance liquid chromatography (HPLC), Thin layer chromatography (TLC), Gas chromatography (GC), Spectroscopic methods like UV spectroscopy, Raman Spectroscopy and its variants, Nuclear magnetic resonance spectroscopy (NMR), Mass spectroscopy, Capillary electrophoresis, Hyphenated techniques that differentiate the samples based on the variation in their chemical profile (11-20). Though physical and chemical methods are amenable for food authentication, in certain instances they fail to give correct results (21). The usage of physical methods is often limited due to their time consuming procedure and need for a skilled expertise. The requirement of an expensive standard and the non-availability of standards for certain botanicals restrict the use of analytical methods in food authentication (22). Molecular methods can compensate these limitations and are dominant over the physical and chemical approaches due to its accuracy, effectiveness and non-dependence on the physical form of the sample (whole or powdered), age, environmental factors, storage and processing conditions, especially for the biological adulterants (23,24).

Molecular methods involve the amplication of one or more regions of genomic DNA using polymerase chain reaction and have a great potential in food authentication due to its sensitivity, rapidity, specificity and simplicity (6). The different PCR based methods used for food authentication and traceability are random amplified polymorphic DNA (RAPD) (25,26), arbitrarily primed PCR (AP-PCR) (27), DNA amplification fingerprinting (DAF) (28), inter-simple sequence repeat (ISSR) (29), directed amplification of minisatellite-region DNA (DAMD) (30), sequence characterised amplified regions (SCAR) (31,32), amplification refractory mutation system (ARMS) (33), simple sequence repeat (SSR) analysis (34,35), species specific PCR (36), single nucleotide polymorphism (SNP) (37) and real time PCR (38).

Apart from these techniques, DNA barcoding, a recently evolved molecular marker

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is gaining acceptance and dominance, as a tool for food authentication and traceability, over the other DNA based methods due to their universality and reliability since last 6 -7 years (Fig. 1).

DNA Barcoding : The concept of DNA Barcoding, proposed and developed by Dr. Paul Hebert, a Canadian Biologist, in animals for species identification, is based on the sequence variation in short nucleotide stretches called "barcodes" between species (39). These barcode regions could act as a species recognition tag by comparing it with the sequences present in a reference database containing sequences of the standardized barcode region of almost all the organisms. If an organism fails to match with any sequence in the database, it could be considered as a possible new species (40). An ideal barcode should be easily amplifiable, amenable to sequencing, exhibit higher interspecies variation than intraspecies variation, easily annotated for evaluation of sequence quality and error detection, recoverable from degraded samples (41). A short 648bp region at the 5' end of mitochondrial cox1 (CO1) gene known as the Folmer region, coding for the cytochrome c oxidase subunit served as the standard barcode in animal kingdom (42). The use of the mitochondrial region in barcoding was dominant over the nuclear genome as it did not exhibit gene duplications common in nuclear genome (43).

Increasing work in the arena of DNA barcoding paved way for the formation of two International collaborations, The Consortium for the Barcode of Life (CBOL) and International Barcode of Life (iBOL), for the progression of DNA Barcoding. CBOL is an organization consisting of more than 200 members representing 50 countries estabilished in May 2004 with the support from Alfred P Sloan Foundation, USA to promote DNA barcoding as a global tool for species identification by compiling sequences in a reference DNA library. iBOL, comprising members from 25 nations was set up in 2014 at Guelph, Ontario with an objective to barcode 5 million specimens and 500,000 estimated species present on earth by 2015 (44). The huge data generated by these two organizations initiated the development of Barcode of Life Database (BOLD), a work bench for the acquisition, storage, analysis and publication of DNA barcode data maintained by the University of Guelph, Ontario, Canada (45).

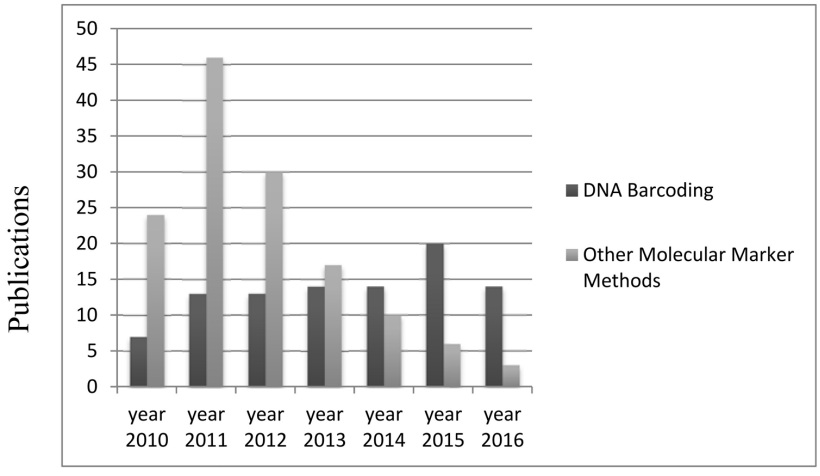

Fig. 1. Publications on DNA barcoding vs.other DNA based methods in food and agricultural commodity authentication.

DNA Barcoding –the Plant Saga : Attempts at plant barcoding using *CO1* gene was a failure due to the low rate of plant species discrimination owing to lack of nucleotide substitutions and high rate of chromosomal rearrangements due to intramolecular recombination in mitochondrial plant genome (46-49). Genome - wide horizontal gene transfer, hybridization and homoplasy also restricted the development of barcoding regions for plants (50). Inspite of these hindrances, regions of chloroplast and nuclear genome were proposed as possible candidates for barcoding in plants. Among the nuclear genomic regions, only the internal transcribed spacer (ITS) of nuclear ribosomal DNA could be used as a barcode owing to lack of universal primers for amplifying the other single copy genes or introns (48) and the technical issues caused by gene duplication in the nuclear genome (51). So focus was shifted to chloroplast genome for barcoding in plants. Regions of chloroplast genome, analogous to mitochondrial genome, sharing its characteristics like conserved gene arrangement, high copy number and availability of universal primers, were proposed as potential candidates as plant barcodes (52). The structural stability, uniparental inheritance and haploid nature of chloroplast genome also facilitated its use as a barcode in plants (53). Internal transcribed spacer of the nuclear ribosomal cistron, some of the coding regions like *rbcL, matK, rpoC1, rpoB* and non-coding regions of the chloroplast genome like *trnH-psbA, atpF-atpH, psbK-psbI, tnnL-trnF* that meet the criteria were proposed as candidate barcodes in plants. In some cases, individual loci failed to serve its purpose. Multi-locus barcode approach was introduced to overcome the same where a phylogenetically conserved easily aligned locus (*rbcL)* is combined with more rapidly evolving variable regions like *matK* or *psbA-trnH* (54). Complementing of these loci will ensure species discrimination and assignment of plants to the correct genus (55).

DNA Barcoding as an Authentication Tool for Food and Agricultural Commodities : DNA barcoding is an efficient marker technique with an important role in certifying food origin, quality of food, safeguarding public health and minimizing food piracy (56, 57). It is based on the analysis of the polymorphic sites in the barcode sequences generated, for the raw materials employed and the food products derived from them (58, 39). These sequences can be compared with the standard sequences deposited in the easily accessible reference databases like GenBank and BOLD to ensure food authenticity and protect consumers from food fraud (58). Increased sensitivity, target DNA sequence diversity, amplification of minute amounts of DNA as in processed products makes DNA barcoding amenable as an authentication tool. Thus, DNA barcoding is widely used for the authentication of medicinal plants and spieces, other agricultural commodities, tea, olive oil, seafood and meat (59-63).

Authentication of Medicinal Plants : The efficacy of herbal medicines mainly depends on the quality of the raw materials used. Unfortunately by default or design many of the herbs mentioned in various pharmacopeia are adulterated taking advantage of the absence of an easy and reliable analytical tool to identify the genuine material from the spurious one. DNA barcoding has now emerged as a very useful approach to authenticate medicinal herbs (Table 1).

Authentication of Spices : Spices are low volume high value commodities traded globally as food flavorant, nutraceuticals,or for medicinal and cosmetic uses. Value added forms of spices like spice powders, crystals, oils and oleoresins. after having lost their morphological diagnostic features are more vulnerable to adulteration than the whole commodity. As in case of medicinal herbs, DNA barcoding based adulteration detection and authentication methods are becoming very popular in traded spices too (Table 2). Adulteration detection can be done at band level or sequence level. In case of band level analysis, the adulterant and genuine product may differ in their amplicon size for a particular gene. The incidence of chilli(*Capsicum annuum* L.) adulteration in traded black pepper (*Piper nigrum*

L.) powder based on the difference in the band size of *trnH-psbA* amplicons was reported (105). *Piper nigrum* samples gave amplicons of size 350bp while adulterated samples (with chilli) gave amplicons of 650bp and 350bp (Fig. 2), respectively for chilli and black pepper.

Authentication of other Agricultural Commodities: Based on the amplification, sequencing success and resolution power, *matK and At103* were proved to be ideal for discrimination of poisonous plants from other useful plants (114).

Canola and saffron oil contamination in olive oil could be detected using DNA barcodes (61). *psbA-trnH* and *matK* primers specific for canola, saffron and olive oil were designed and successfully amplified. These primers were able to detect 5% and above adulteration in olive oil samples. Further confirmation was done by sequencing the amplicons produced and comparing it with the barcodes deposited in reference databases.

A study on barcoding of herbal teas using barcoding loci *rbcL* and *matK* revealed that 35% of the samples generated barcodes for unlisted ingredients on the brand label. *Matricaria recutita* was the most common unlisted ingredient found in seven herbal tea products. Four herbal teas yielded *Camellia sinensis* barcodes although it was not listed on the label. Barcode from a herbal tea sample was similar to *Poa annua* and four products generated barcodes similar to plants of parsley family (115).

DNA barcoding was employed to authenticate the plants used in the preparation of Chinese "cooling beverage",obtained from single or mixture of plants (116). *rbcL, matK, psbA-trnH* and ITS loci were used in the differentiation of Kudidan (*Elephantopus scaber*) from its substituent *Elephantopus tomentosus*; Ludougen (*Pandanus tectorius*) from its adulterant (*Pandanus austrosinensis*); Shepaole (*Rubus reflexus*) from *Rubus parvifolius* and Xiangsizi (*Abrus precatorius*) from *Abrus cantoniensis*. *psbA-trnH, ITS, matK* and *rbcL* alignment depicted, 12-46, 9-70, 1-7 and 1-5 polymorphic sites, respectively that served as markers to distinguish between these genuine and adulterant species. Though *rbcL* had the least number of polymorphic sites, only it could be successfully amplified in all the 4 traded samples of the cooling beverage tested. *rbcL* revealed that traded samples of Kudidan, Ludougen and Xiangsizi as genuine material while Shepaole was substituted (116).

Traceability of *Lycium barbarum,* a nutritious food from other species like *Lycium chinense* and *Lycium ruthencium* was done using ITS2 barcode. BLAST1, NJ tree and nearest distance analysis using the ITS2 sequences could successfully discriminate between these three species (117).

The increasing demand for non-Camellia teas has paved way for its substitution with adulterants resulting in allergic reactions. BLASTN and phylogenetic analysis based on

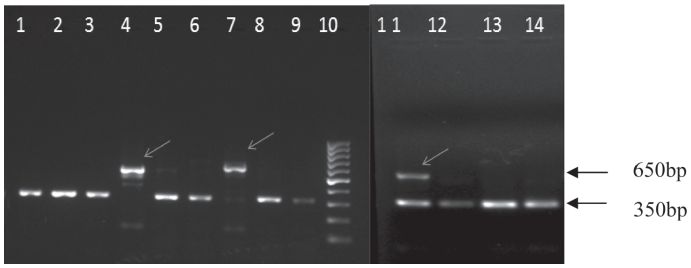

Fig. 2. Amplification of *psbA-trnH* locus (Lanes 1-3 -*Piper nigrum,* lane 4- *Capsicum annuum,* lane 5-Market sample1, lane 6- Market sample 2, lane 7- Market sample 3, lane 8- Market sample 4, lane 9- Market sample 5, lane 10 -100bp ladder, lane 11-Market sample 6, lane 12- Market sample 7, lane 13- Market sample 8, lane 14 -Market sample 9.

Table 1. Application of DNA barcoding in adulteration detection of medicinal plants

Application	Barcoding loci	Reference
Authentication of medicinal plants of Polygonaceae	*trnH-psbA*	(64)
Distinction between *Radix astragali* and its adulterants	ITS, *matK*	(65)
Authentication of *Taxillus chinensis*	ITS	(66)
Assessment of species admixture in *Phyllanthus amarus*	*trnH-psbA*	(67)
Authentication of *Ruta graveolens*	ITS	(68)
Differentiation of *Lonicera japonica* from its adulterant species	*trnH-psbA*	(69)
Molecular authentication of *Sabia parviflora* and its adulterants	*matK, rbcL, trnH-psbA*	(70)
Identification of *Gentianopsis paludosa* from its adulterants	ITS	(71)
Distinguishing medicinal plant *Paris polyphylla* from its Adulterant *Valeriana jatamansi*	*trnH-psbA*	(72)
Authentication of Black cohosh dietary supplements	*matK*	(73)
Authentication of commercialized medicinal plants in Southern Morocco	*rpoC1, matK*, *trnH-psbA*, ITS	(74)
Identification of *Solanum lyratum* from its substituent *Aristolochia mollissima*	ITS, *matK, rbcL, trnH-psbA, trnL-trnF*	(75)
Identification of *Dipsacus speroides* from three other species of genus *Dipsacus.*	ITS2	(76)
Authentication of *Sedum sarmentosum* from its adulterants	ITS2	(77)
Authentication of herbal materials	ITS2	(78)
Distinguishing *Boerhavia diffusa* from its adulterants	ITS	(79)
Differentiation of *Hemidesmus indicus* from its adulterant *Decalepsis hamiltonii*	ITS2	(80)
Authentication of natural health products	ITS, *rbcL*	(81)
Identification of tobacco seized from water pipes	*matK, rbcL*	(82)
Authentication of medicinal plants of Fabaceae	*trnH-psbA*	(83)
Discrimination of *Scutellaria baicalensis* from its adulterants	*trnH-psbA, rbcL*	(84)
Authentication of dietary supplement saw palmetto	*matK, rbcL*	(85)
Identification of processed medicinal materials in South Africa	*matK, rbcL*	(86)
Authentication of *Salvia divinorum* samples	*rbcL, trnL-trnF*	(87)
Detection of contamination & substitution in North American health products	*rbcL,* ITS	(59)
Differentiation of *Gentian a*s pecies traded as Guanlongdan from its adulterants	*rbcL, matK, trnL-trnF, trnH-psbA,* ITS	(88)
Discrimination of medicinal plant *Isatis indigotica* from its adulterants	ITS2, *rbcL, trnL-F*	(89)
Authentication of drugs used in traditional Chinese medicine	*rbcL*	(90)
Authentication of *Gingko biloba* dietary supplements	*matK*	(91)
Authentication of *Cassia* species used in traditional Indian medicine	*rbcL, trnH-psbA*	(92)
Molecular authentication of commercially sold medicinal Plants in Manila	*matK, trnH-psbA*	(93)
Authentication of medicinal materials sold in Dali fair	ITS2, *trnH-psbA*	(94)
Detection of adulteration in the drug trade of “Bala” drugproducts	*trnH-psbA*, ITS2	(95)
Authentication of *Swertia chirayita* and its adulterant species	ITS	(96)
Authentication of *Sida cordifolia* herbal products	*trnH-psbA*, ITS2	(97)
Authentication of commercial processed Glehniae Radix	ITS2	(98)
Authentication of medicinal plants used in herbal medicine in Brazil	*rbcL, matK,* ITS2	(99)
Assessment of adulteration in health products of *Cassia*, *Senna* and *Chamaecrista* in South India	ITS2	(100)
Discrimination between toxic aristolochiaceous and non-aristolochiaceous plant materials	ITS2, *trnH-psbA*	(101)
Survey of commercial Rhodiola products	ITS2, *trnH-psbA*	(102)
Molecular identification of Arisaematis Rhizoma and Pinelliae Tuber from its adulterants	*matK, rbcL*	(103)
Authentication of medicinal plant raw drugs used in Ayurvedic medicine	*rbcL*	(104)

Table 2. Application of DNA barcoding in adulteration detection of spices

Application	Barcoding loci	Reference
Identification and traceability of different spices like mint, sage, thyme, origanum and basil	*trnH-psbA, matK*	(60)
Authentication of *Ilicium verum* (star anise) from its adulterants	*trnH-psbA*	(106)
Traceability of commercial saffron	ITS	(107)
Detection of chilli adulteration in traded black pepper	*trnH-psbA, rbcL*	(105)
Discrimination of *Cinnamomum verum* from *C. cassia*	*rbcL*	(108)
Detection of adulterants in saffron	*trnH-psbA*	(109)
Tracing out adulterants in traded turmeric powder	ITS, *rbcL*	(110)
Detection of adulterants in traded nutmeg mace	*trnH-psbA*	(111)
Detection of adulteration in saffron	*matk, rbcL*	(112)
Authentication of saffron by mini-barcodes	*matK*, ITS1, ITS2	(113)

barcoding loci viz. *rbcL, matK, psbA-trnH* and ITS2 could identify non-Camellia teas from the samples screened thus providing a way to effectively label the commercially available samples and ensure safety to consumers (118).

DNA barcoding was used to solve a dispute in the international trade of roasted barley tea. Roasted barley tea consignment imported from China was rejected due to substitutions. Barcoding of the rejected barley tea samples using *rbcL, matK, psbA-trnH* and ITS2 loci revealed that out of the 13 batches of samples tested, 1 batch was substituted with *Morus* species. Out of the remaining 12 batches, 2 batches had only *Hordeum vulgare* while 10 had *H. vulgare* admixed with *Morus* spp*., Triticum* spp. *Avena sterilis* and *A. fatua* (119).

DNA barcoding technique was adopted to identify the source plants composition in processed honey using *rbcL* and *psbA-trnH* loci. BLAST analysis showed that the four honey samples studied are obtained from 39 plant species of genus such as *Castanea*, *Quercus*, *Fagus* and other herbal taxa. One out of the four samples studied also showed traces of genomic DNA from *Atropa belladonna,* a toxic plant thereby conforming the applicability of barcoding in certifying the food safety of commercial honey (120).

Authentication of seafood and meat : Efficiency of DNA barcoding in seafood traceability has resulted in its adoption as a method for authentication of fish based commercial products by the US Food and Drug Administration (121). Barcode based seafood authentication has been successful due to the availability of more than 70,000 reference sequences in the database, Fish Barcode of Life Initiative (FISH-BOl) (57). Many authors have reported mislabeling, substitution and adulteration of seafood, processed seafood and their byproducts like fish fillets using *cox1* gene based barcoding (122-126). Meat adulteration in US markets could also be traced using *cox1* based barcoding (63, 127) (Table3).

Conclusion

Globalisation has resulted in increased global trade accompanied by a rise in the unscrupulous practices of adulteration to attain economic gain resulting in eroding the perceived biological value and quality of the product besides corroding public faith. Food quality control is an essential prerequisite to safeguard the consumer's interests and health. DNA barcoding has been used an ideal tool to check food quality due to its simplicity, easiness, rapidness and sensitivity. It may soon emerge as a routine quality test for food authetication and traceability across the globe. The polymorphic sites found in the barcoding loci can be exploited to synthesize specific primers for developing diagnostic kits that may be handy for the food regulatory agencies in ensuring the quality of the traded food and agricultural commodities at the

Table 3. Applications of DNA barcoding in seafood and meat authentication

Application	Target gene	Reference
Identification of smoked fish products	*CO1*	(128)
Detection of market substitution in North American seafood	*CO1*	(129)
Identification of shark and ray fins using DNA barcoding	*CO1*	(130)
Detection of mislabeling in Amazonian commercial fish	*CO1*	(131)
Substitution of shark seafood products	*CO1*	(8)
Revelation of mislabeling in commercial fish products in Italy	*CO1, cytb*	(132)
Revelation of high rate of mislabeling in commercial fresh water catfish in Brazil	*CO1*	(133)
Revelation of high incidence of fish species misrepresentation and substitution in South African market	*CO1*	(134)
Market place substitution of Atlantic salmon in place of Pacific salmon in Washington	*CO1*	(135)
Revelation of market substitution of fish in Canada	*CO1*	(136)
Species authentication of catfish	*CO1*	(137)
Authentication of commercialized crab meat in Chile	*CO1*	(138)
Authentication of commercial seafood products	*CO1*	(139)
Species identification of some fish processing products in Iran	*CO1*	(140)
Detection of market substitution in salted cod fillets and battered cod chunks	*CO1*	(141)
Detection of mislabeled commercial fishery by-products in Philippines	*CO1*	(123)
Authentication of exploited grouper fish species	*CO1*	(142)
Authentication of processed and raw tuna from Indonesian markets	*CO1*	(143)
Revelation of mislabeling in Egyptian fish fillets	*CO1*	(144)
Detection of improper labeling and supersession of crab food served by restaurants in India	*CO1*	(145)
Authentication of snappers of West Atlantic	*CO1*	(146)
Revelation of commercial and health issues in ethnic seafood sold in the Italian market	*CO1*	(147)
Authentication of Porgies fish species of commercial interest on the international market	*CO1*	(148)
Governmental regulatory forensic program for identification of commercialized seafood in South Brazil	*CO1*	(124)
Detection of fish mislabelling and substitution in South Africa	*CO1*	(149)
Revelation of high substitution and mislabeling of croaker fillets in Brazil	*CO1*	(150)
Unmasking seafood mislabeling in US markets	*CO1*	(125)
Labelling accuracy in seafood retailers in Tasmania, Australia	*CO1*	(151)
Seafood identification using DNA barcoding revealed market substitution in Canadian seafood	*CO1*	(62)
Revelation of mislabeling of processed flat fish products in southern Italy markets	*CO1*	(152)
Authentication of heavily processed fish products using DNA mini barcoding system	*CO1*	(153)
Detection of mislabeled seafood products in Malaysia	*CO1*	(154)
Government commissioned authentication of fish products in Taiwan	*CO1*	(155)
Identification of species in ground meat products sold in US market	*CO1*	(63)
Mislabeling in Indian seafood	*CO1*	(156)
Revelation of mislabeling of game meat species in US markets	*CO1*	(157)
Revelation of fraud in commercial yak jerky sold in China using DNA barcoding	*CO1,*16SrRNA	(158)
Identification of common aquatic products and the supervision of its market trade in Central China	*CO1*	(159)
Revelation of mislabeling of imported fish productsin Nansha port of China	*CO1*	(160)
Revelation of mislabeling in seafood sold in Portuguese supermarket	*CO1*	(161)

port of origin/destination or in domestic whole sale or retail outlets.

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NEWS ITEM

SCIENTIFIC NEWS

PSLV-C35 Launched Eight Satellites using Single Flight: ISRO's Polar Satellite Launch Vehicle (PSLV) successfully launched the SCATSAT-1 Satellite (371 kgs) along with seven co-passenger satellites on September 26, 2016 from Satish Dhawan Space Centre SHAR, Sriharikota. This is the thirty sixth consecutively successful mission of PSLV. The total weight of all the eight satellites carried on-board PSLV-C35 was 675 kg. PSLV-C35 is the first PSLV mission to launch satellites carried onboard into two different orbits. This PSLV mission was the longest of the missions conducted till date and was completed in 2 hours 15 minutes and 33 seconds after lift-off.

Human hair served as cathodes for solar cells: Researchers at the Indian Institute of Science Education and Research (IISER) in Kolkata have used human hair to produce cost-effective, metal-free cathodes for use in solar cells. This is the first instance where a bio-waste-derived electrode has been used as cathode in a quantum dot sensitised solar cell device. While metal-free cathodes produced in the past have not performed as well as the traditional metal-based ones, the performance of graphitic porous carbon cathode was produced was at par with metal-based cathodes.

NOBLE PRIZE AWARDEES IN SCIENCE-2016

Nobel prize in Medicine: The 2016 Nobel Prize in Physiology or Medicine has been awarded to Dr.Yoshinori Ohsumi. The Nobel Committee said the Prize has been awarded to him "for his discoveries of mechanisms for Autophagy." The professor is currently at the Tokyo Institute of Technology. Announcing the prize in Stockholm, the Nobel Committee said that the cell biologist "discovered and elucidated mechanisms underlying autophagy, a fundamental process for degrading and recycling cellular components."

Nobel Prize in Chemistry: The Nobel Prize in Chemistry for 2016 has been awarded to Dr.Jean-Pierre Sauvage, Sir Dr.J. Fraser Stoddart and Bernard L. Feringa for developing molecular machines. Announcing the Prize on Wednesday in Stockholm, a statement from the Royal Swedish Academy of Sciences said, "The development of computing demonstrates how the miniaturisation of technology can lead to a revolution. The 2016 Nobel laureates in Chemistry have miniaturised machines and taken chemistry to a new dimension". "They have developed molecules with controllable movements, which can perform a task when energy is added. Molecular machines will most likely be used in the development of things such as new materials, sensors and energy storage systems.

Noble prize in Physics: The Nobel Prize in Physics for 2016 has been awarded to Dr.David J. Thouless, and the other half to Dr.F. Duncan M. Haldane and Dr.J. Michael Kosterlitz for theoretical discoveries of topological phase transitions and topological phases of matter. Announcing the Prize in Stockholm, a statement by the Royal Swedish Academy of Sciences said that "This year's Laureates opened the door on an unknown world where matter can assume strange states. They have used advanced mathematical methods to study unusual phases, or states, of matter, such as superconductors, superfluids or thin magnetic films.

Calcium supplements may damage Heart: Taking calcium in the form of supplements may raise the risk of plaque buildup in arteries and heart damage, although a diet high in calcium-rich foods appears to be protective, scientists have stated. After analyzing 10 years of medical tests on more than 2,700 people, researchers at Johns Hopkins University School of Medicine, said the results add to growing scientific concerns about the potential harms of supplement. "Our study adds to the body of evidence that excess calcium in the form of supplements may harm the heart and vascular system," said Erin Michos, from Johns Hopkins Medicine. Previous studies have shown that "ingested calcium supplements - particularly in older people - do not make it to the skeleton or get completely excreted in the urine, so they must be accumulating in the body's soft tissues," said nutritionist John Anderson, from University of North Carolina.

Regeneration of Monkey Hearts by Lab-grown stem cells: In a step forward for organ regeneration, stem cells grown from a single monkey's skin cells revitalized the damaged hearts of five sick macaques. The experiment builds towards the goal of providing

a vast and uncontroversial source of rejuvenating cells to transplant into heart attack victims. This would obviate the need to harvest stem cells from embryos or from transplant recipients themselves. The team used so-called induced pluripotent stem cells (iPSCs).

These are created by stimulating mature, already specialized cells — such as a skin cell. Adult heart stem cells have already been experimentally used in heart attack victims. And therapy with embryonic stem cells has shown promise in treating severe heart failure. But the Japanese team said theirs was the first study to use iPSCs to fix heart damage. Human iPSCs have long been touted as a promising source of cells for heart repair. In the monkey trials, the team chose a molecule in an immune-system cell that was a match in both donor and recipients, to stop the body's defence system identifying and reacting to the "intruder" cells. "We still have some hurdles, including the risk of tumour formation, arrhythmias, cost, etc." the authors of Japan's Shinshu University stated.

Apes think far more like humans than previously believed: Kind of understanding that were thought to exist only in humans might also be shared by apes, according to the finding of new research. Research with chimpanzees, bonobos and orangutans, however, suggests that our distant relatives might be able to know the same thing. "This cognitive ability is at the heart of so many human social skills," said Christopher Krupenye of Duke. The result of the test - referred to as the "false belief test" - were similar to those found in humans that are under the age of two. The research was conducted by scientist at Duke University, Kyoto University and the Max Planck Institute for Evolutionary Anthropology.

Human life span cannot be extended any more: The peak of human life span has already been reached and instead of wasting resources trying to extend life, research should concentrate on extending health span, the duration of old age spent in good health, says a new study published in Nature. Demographers as well as biologists have contended there is no reason to think that the ongoing increase in maximum lifespan will end soon. But our data strongly suggest that it has already been attained and that this happened in the 1990s."

HIV cure is on the verge says British scientists: British scientists are on the verge of confirming a treatment for HIV, an incurable virus that leads to deadly AIDS disease, if a new treatment trial proves successful. A British man with HIV hopes to become the first in the world to be cured of the disease by using the pioneering new therapy designed to eradicate the virus, 'The Sunday Times' reported. It is the first therapy created to track down and destroy HIV in every part of the body — including in the dormant cells that evade current treatments. If successful, it offers hope of an irreversible cure for HIV and could save millions of pounds spent on drugs. This is one of the first serious attempts at a full cure for HIV. This is a huge challenge and it's still early days but the progress has been remarkable.

CSIR developed New Herbal drug for management of Diabetes: Scientists at the Centre for Scientific and Industrial Research awarded yesterday for developing BGR-34, an Ayurvedic anti-diabetic drug, said that the drug has been found to be successful in controlling blood sugar. Developed after about four years of research at CSIR labs in Lucknow, the drug was found to reduce the HbA1c (a type of haemoglobin, its level reflects how well the body is controlling diabetes) levels from 7.8% to 7.3% in diabetic patients who were given a daily dose of BGR-34 for three months. Scientific studies have shown that complications related to diabetes can be delayed or prevented by keeping HbA1c level below 7%. Post-prandial blood sugar levels reduced from 204 to 194 mg/dl. In healthy adults it should be less than 180 mg/dl of blood. The human trial for the drug was conducted on 48 adults with diabetes.

Horses can communicate with humans: Horses can learn to communicate with humans and express their feelings and opinions, a new study has claimed. Researchers from Norwegian Veterinary Institute trained horses by offering slices of carrot as an incentive to touch a board with their muzzle to indicate if they wanted to wear a blanket. The horses' requests matched the weather, suggesting it was not a random choice.

Researchers hope that the method could be used to ask horses more questions. They believe that ordinary horse owners will be able to train their horses in this way. It was taught to tell the difference between different symbols on the board - blanket on (horizontal bar), blanket off (vertical bar) and no change (blank). Finally, the horse was taught to associate a particular action with the symbols on the board.

Chink in E.coli armour: It was stated that E. coli bacteria more susceptible to host immune

response. The researchers at IIT, Hyderabad, have found a potential way of preventing the bacterial surface-associated polysaccharide — capsular polysaccharide (CPS) — from attaching on the surface membrane and forming a protective encapsulation of the bacteria, thus making the E. coli vulnerable to attack by the host's immune system. The CPS is synthesised by the bacteria and exported to the surface to offer protection by evading the host immune response. Surface-association of CPS also offers impermeability to antibiotics, thus establishing infection in the host. Certain surface-associated bacterial proteins help in the attachment of CPS on the bacterial surface. The CPS is not the same in all the E. coli strains but varies. In all, there are 80 such capsular polysaccharides. They have modelled the 3D structures and developed an organised repository of 72 CPS varieties.

Treatment for Residual Glioma Cancer cells: Photodynamic therapy for treating residual cancer cells of a high-grade brain tumour (glioblastoma) has been reported recently. Photodynamic therapy uses a photosensitive drug that becomes active under the action of light and converts molecular oxygen into reactive oxygen species that kill cancer cells. Scientists at the Amrita Institute have turned to nanotechnology and used light in the near-infrared region to achieve better results. Light in the near-infrared region can penetrate to about 0.8 cm into body tissues. The drug encapsulated in a nanoparticle has peptides functionalised on its surface and is selectively absorbed only by cancer cells. The nanoparticles containing the drug have better ability to kill cancer cells as they absorb three times more light in the near IR region than the free drug.

Electric bandage developed by NASA for wound healing: NASA has developed a new high-tech material that uses electricity to significantly promote healing of injured wounds. In conditions of non-Earth gravity, human blood displays behaviour quite different from that on Earth. Wounds are likely to heal much more slowly and considering the survival risks and the cost of space missions, healing wounds as fast as possible is crucial.

The new material generates a small amount of electricity when interacting with another surface, including human skin. The material, called polyvinylidene fluoride (PVDF) has numerous possible applications, including wound healing. It is proven that wounds tend to heal much more quickly if small amounts of electricity are applied to the surrounding tissue. If the PVDF fibres are aligned correctly, cells on a wound use it as a scaffold, helping the wound to heal faster.

POST DOCTORAL FELLOWSHIPS

IIT Madras Post doctoral fellowships: IIT Madras invites applications for PDFs in it's different departments. The applications should be sent through email to drresearch@iitm.ac.in along with the relevant documents. For more information please visit website https://www.iitm.ac.in/ content/ post-doctoral-fellowship-iit-madras.

SERB-National Post Doctoral Fellowship (N-PDF): SERB invites applications for N-PDF. The call for applications for SERB-N PDF will be notified twice a year through the websites www.serbonline.in and www.serb.gov.in. The application form along with a research proposal highlighting the objectives of the research work to be undertaken should be submitted online through the website www.serbonline.in. The fellows will work under a mentor, and it is hoped that this training will provide them a platform to develop as an independent researcher.

Ramanujan Fellowship: The fellowship is meant for brilliant scientists and engineers from all over the world to take up scientific research positions in India, i.e. for those scientists who want to return to India from abroad. The fellowships are scientist-specific and very selective. For more information please visit website **http://www.serb.gov.in/ rnf.php**

Start-Up Research Grant (Young Scientists): Start-up grant for Young Scientists is restructured **w.e.f. 1st September, 2015** into two new schemes: Early Career Research Award (ECRA) and National Post-Doctoral Fellowship (NPDF). For more information please visit website http:/ /www.serb.gov.in/srg.php

SERB Overseas Postdoctoral Fellowship: SERB Overseas Postdoctoral fellowship (SERB-OPDF) aims to build national capacity in frontier areas of Science and Engineering, which are of interest to India by providing postdoctoral fellowship for a period of one year extendable to one more year subject to good performance. The Program admits candidates in identified areas and sends them to top institutions around the globe, other than USA and also to institutions where internationally acclaimed scientists are working. For more information please visit website http://www.serb.gov.in/opf.php

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ANNOUNCEMENTS

2nd AP Science Congress: The second Andhra Pradesh Science is being organized at Vijayawada jointly by Andhra Pradesh Akademi of Sciences, Dr.NTR University of Health Sciences, Acharya Nagarjuna University and Krishna University, with a focal theme of "Science and Technology for Health" at PB Siddhartha College Auditorium on 7-9 November, 2016. It is being inaugurated by Hon'ble Chief Minister of Andhra Pradesh, Sri.N. Chandrababu Naidu and Hon'ble Union Minister for Urban Development, Housing, Poverty Alleviation, Parliamentary Affairs & Ministry of I&B, Sri.M.Venkaiah Naidu. For details of participation, contact through apsc2016vijayawada@gmail.com

104th Indian Science Congress: The pilgrimage city, Tirupati has been chosen to host the 104th Indian Science Congress meeting to be held during January 3 to 7, 2017 with a focal theme of "Science and Technology for National Development, which will be inaugurated by Prime Minister Narendra Modi on the sprawling Sri Venkateswara University campus. As many as 10,000 delegates, who are directors and office-bearers of various science organisations, are expected to converge on the city for the mega event. Apart from the main event, there will be a women's science congress and children's science congress. For further details, one may contact through email - drsvbr.acas@gmail.com. The 104th Indian Science Congress, will witness a host of scientific luminaries from countries across the world, said Indian Science Congress Association general president Prof.D. Narayana Rao. Prof. Rao said that nine Nobel Laureates from the U.S., Japan, France, Israel and Bangladesh would be attending the event and share their experiences. In addition to this, a huge contingent of 200 scientists from foreign nations, 10,000 scientists representing various national laboratories, faculty and research scholars from Indian Universities and several others will take part in the event to exchange their views on a range of scientific issues, both on national and international level, he added. Plenary sessions are being organised in tune with the vision of Prime Minister Narendra Modi, to transform India through science and technology.

MS in Pharmacy from USA

1st semester at Alliance - JNTUH in India and remaining courses & research in USA at the University of the Pacific, California, USA.

University of the Pacific, USA has entered into collaboration with JNTUH & Alliance Institute, India, for offering Masters (MS) program in Industrial Pharmaceutics. In this program students take courses in the first semester at Alliance-JNTUH and after successful completion of first semester at Alliance and fulfilling admission, TOEFL and visa requirements, students can go to USA to complete remaining courses and research at Thomas J Long School of Pharmacy and Health Sciences, University of the Pacific. Upon successful completion of the requirements, University of the Pacific will award Master's degree.

If students fail to meet University of the Pacific admission/visa requirements, they have an option to continue their course and research work at Alliance -JNTUH or do research work at the Pacific to fulfill requirements for MS degree in India.

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