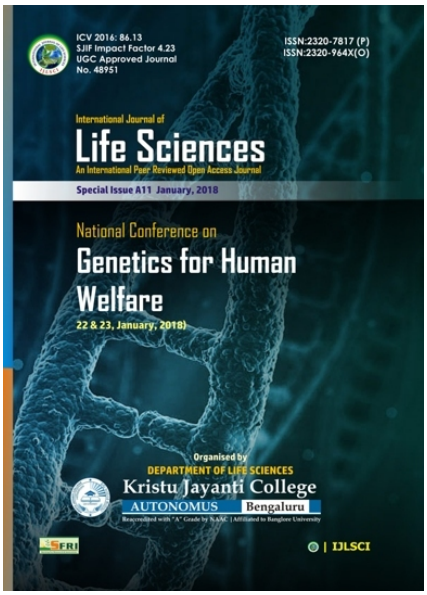
ICV 2016: 86.55
UGC Approved Journal No. 48951
Cosmos Impact Factor 4.369
SJIF Impact Factor 4.23

ISSN:2320-7817 | eISSN:2320-964X
Special Issue, A 11, January 2018

INTERNATIONAL JOURNAL OF Life Sciences

AN INTERNATIONAL PEER REVIEWED OPEN ACCESS JOURNAL



National Conference on

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Publisher and Owner: **Dr. Arvind Chavhan,** Published from IJLSCI, 46, Guruvandan, Jawahar Nagar, VMV Road Amravati -444604, India

Printed at IJLSCI, 46, Guruvandan, Jawahar Nagar, VMV Road Amravati -444604, India

Managing Editor: Dr.Arvind Chavhan, Assistant Professor & Head, Department of Zoology, D.B. College, Bhokar, Nanded, India

Indexing:

Index Copernicus, Google Scholar, OCLC WorldCat, INNOSPACE (SJIF), CiteFactor, ROAD, OAJI, AcademicKeys, J-index.net, ScholarSteer, ResearchBible, DRIJ, DIIF, SIS, SIS, ASI, Directory of Science

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IJLSCI

ISSN:2320-7817(p) | 2320-964X(o)

INTERNATIONAL JOURNAL OF LIFE SCIENCES

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ISSN:2320-7817(p) | 2320-964X(o)

INTERNATIONAL JOURNAL OF LIFE SCIENCES

Special Issue A11 January; 2018

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International Journal of
Life Sciences
International Peer Reviewed Open Access Refereed Journal

Special Issue A 11: January 2018
UGC Approved Journal No 48951

Original Article Open Access

Cytogenetic analysis in young people with Epilepsy disorder- A preliminary investigation

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Manuscript details:

Available online on
http://www.ijlsci.in

ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

Editor: Dr. Arvind Chavhan

Cite this article as:
Venkatesan R, Mohamed Ansar Ali B, Sasikala K (2018) Cytogenetic analysis in young people with Epilepsy disorder- A preliminary investigation, *Int. J. of. Life Sciences*, Special Issue, A11: 1-5.

ABSTRACT

Epilepsy is one of the most common neurological diseases worldwide. The present study was carried out to determine the chromosomal aberrations in peripheral blood samples of 20 patients with epileptic seizures as confirmed by their Electroencephalogram (EEG) reports. Subjects included for the study included 10 patients below the age group of 20 and 10 patients above the age group of 20. The majority of aberrations were mostly observed in patients with age group of ≥20 yrs old.

Key words: Epilepsy, Neurological disorder, Chromosome aberrations

INTRODUCTION

Epilepsy is a neurological disorder that affects people all over the world. It is characterized by a tendency to recurrent seizures and is defined by two or more unprovoked seizures. Research on epilepsy conducted worldwide estimated that it has a mean prevalence of approximately 8.2 per 1000 of the total population (Brodie and Schacther, 2001). Globally 50 million people suffer from epilepsy which is the most common neurological disorder affecting children in the developing world (Pal *et al*., 1998). Studies in developed countries suggest that the incidence of epilepsy is around 50 per 100,000 of the general population, where as in developing countries it is around 100 per 100,000 (Fact sheet 168). The occurrence of epilepsy has been assessed in several populations worldwide (Annegers, 2004; Forsgren *et al*., 1996; Haerer *et al*., 1986; Hart *et al*., 1990; Hauser *et al*.,1991, 1993,1998; Juul-Jensen and Ipsen,1975; Sidenvall *et al*., 1996; Wallace *et al*.,1998). The rate of incidence of epilepsy tends to be high in early child hood (200 per 100,000 person-years), low (25- 40 per 100,000 person-years) between 20 and 50 years of age, and high after 50 years of age (Annegers, 2004; Forsgren *et al.,*1996; Hauser *et al.,*1993; Wallace *et al.,*1998). The overall prevalence proportion of epilepsy has been estimated to be 0.5 - 0.9% (Annegers, 2004; Forsgren *et al*.,1996; Haerer

et al.,1986; Hart *et al*.,1990; Hauser *et al*.,1991,1998; Juul-Jensen and Ipsen,1975; Sidenvall *et al*.,1996). About 6% of patients suffering from epilepsy with mental retardation carry chromosome aberrations (Singh *et al*., 2002).

MATERIALS AND METHODS

A total of 20 young patients with epilepsy were selected for this study using electroencephalogram (EEG) reports. The objectives of this study were explained to patients and their informed consent was obtained before including them for this study. The EEG is the depiction of the electrical activity occurring at the surface of the brain. This activity appears on the screen of the EEG machine as waveforms of varying frequency and amplitude measured in voltage (specifically microvoltage). The intravenous blood was collected aseptically using heparin from 20 patients suspected for seizure disorder. The patients were classified into two groups based on their age (Group I <20 yrs old and Group II ≥ 20 yrs old) to check for manifestation of any age-related changes in such patients. Age is a variable which is an important determinant for epilepsy risk. Age, as a surrogate of brain maturation, is a determinant of the specific characteristics of the seizure disorder in those with epilepsy, and age-related changes in these manifestations can be identified (Hauser,1992). Each group thus consisted of 10 patients.

Chromosomal aberration analysis was carried out by following standard procedure (Hoyos *et al*., 1996). Briefly, 0.5 ml of whole blood was added to 5.0 ml RPMI 1640 medium (Hyclone, USA), supplemented with 20% fetal bovine serum (PAA Laboratories, Austria), 2mM L-glutamine (Himedia, India), 1% streptomycin-penicillin antibiotic (Himedia, India) and 0.2 ml of Gibco™ phytohemagglutinin (ThermoFischer Scientific, India).

The mixture was incubated at 37°C for 72 hours. After 71 hours, the cells were treated with 0.01% Colchicine (Himedia, India) to arrest cells in mitosis. Lymphocytes were harvested upon the completion of 72 hours by centrifuging the cells at 1800 rpm for 7 minutes. About 6mL of pre-warmed (37°C) hypotonic solution (KCL 0.075 M) was added and left aside for 20 minutes at room temperature. After removing the hypotonic solution by centrifugation, the cells were fixed in Carnoy's fixative. Slides were prepared and stained in 2% Giemsa stain. For the chromosomal aberration analysis, 100 well spread metaphase plates were examined per subject under a microscope (100X) to identify numerical and structural chromosomal aberrations (Hoyos *et al*., 1996).

RESULTS

Table 1 shows the physical characteristics and chromosomal aberrations observed in the patients of the present study. Patients of both age groups showed both structural and numerical aberrations in different chromosomes. But the frequency was higher in Group II patients. Structural aberrations included deletions in long and short arms of chromosomes (5p-,7p-,14q-,15q-) and satellite structures in 13, 15 and 22nd chromosomes. Numerical aberrations such as trisomies observed for chromosomes 13 and 21.

In the present study almost all the patients exhibited tonic-clonic type of seizures. During the clonic phase of the seizures, the EEG derived from subdural electrodes overlying the motor strip always shows a polyspike wave pattern.

DISCUSSION

Epilepsy is the most common and challenging neurological disorder. This chronic disorder affects a patients' life by limiting his / her social, physical and emotional functions resulting in a poor quality of life. Improving an epileptic's Heath Related Quality of Life (HRQoL) is recognized as an essential component of the management of patients with epilepsy (Jacoby and Faker 2008, Privitera and Ficker , 2004). In the present study almost all the patients exhibited tonic-clonic type of seizures. When seizures occur during the childhood or in the third decade of life, generalized tonic-clonic are the most common together with partial simplex or partial complex seizures, but myoclonic, atonic and absence with tonic seizures have been reported (Singh *et al.,*2002).

An important application of EEGs is for the study of epileptic patients, in which deviations from the "normal" patterns help to classify epilepsies and eventually to localize the epileptic focus. Focal clonic seizures have been defined as series of myoclonic contractions occurring at regular intervals, typically in the range of 0.5± 5 Hz (Hamer *et al.,* 1999).

Table 1: Patients' health status and chromosome aberrations

Case. No	Gender/ age	Type of Seizure	Complaints	Life style factors	Chromosome aberrations
N001	15/F	Tonic-Clonic	Persistent cough Headache and tiredness	-	46, XX
N002	30/M	Tonic- Clonic	Wheezing trouble, Joint pain and severe headache	-	46, XY (5p-)
N003	28/F	Tonic- Clonic	-	-	46,XX
N004	22/M	Tonic	Headache and tiredness	-	46, XY
N005	17/F	Tonic- Clonic	Speech problem, Severing, Numbness Fearing and sleeping disturbance	-	46,XX
N006	14/M	Tonic	-	-	46,XY
N007	29/M	Tonic- Clonic	-	-	46, XY (7p-)
N008	16/F	Tonic-clonic	High Blood pressure, persistent cough and Joint pain	-	46, XX
N009	27/F	Tonic-clonic	Wheezing trouble and persistent cough	-	46, XX
N010	17/F	Tonic-clonic	-	-	46, XX
N011	14/M	Tonic-clonic	Spinal cord pain and Joint pain	-	46, XY (7p-)
N012	13/F	Tonic-clonic	-	-	46, XX
N013	20/M	Tonic-clonic	Unconsciousness	-	47, XY (5p-, 13S$^+$ and 22S$^+$), trisomy 21
N014	25/M	Tonic-clonic	Unconsciousness', Headache, Joint pain and weight loss	-	46, XY
N015	13/F	Tonic -clonic	Joint pain and Headache	-	46, XX
N016	22/M	Tonic clonic	Headache, unconsciousness	-	47, XY(14q-) trisomy 13
N017	24/ F	Tonic clonic	Headache	-	46, XX (14q-)
N018	15/ M	Tonic clonic	-	-	46, XY (15q-)
N019	22/M	Tonic clonic	Head ache and unconsciousness	-	46, XY
N020	18/M	Tonic clonic	-	-	47, XY (15S$^+$, 22S$^+$) trisomy 21

Focal clonic seizures are always associated with a Polyspike wave pattern in the EEG of the primary motor area (frequency range 1.6±3.4 Hz). The clonic phase started at a frequency between 1.9 – 3.4 Hz in the present study of patients. The muscular contractions of the clonic phase are a response to brain activity that can only be established when brain oscillations are slow enough to be followed by the muscles. In the present study both structural and numerical chromosomal aberrations were observed in patients.

Structural chromosome aberrations present included deletions in chromosomes 5, 7, 14, 15, and satellite

structures in chromosomes 13, 15, and 22. Seizures onset ranging from neonatal period to 7 years are reported in patients affected with deletion of the long arm of chromosome 7. Febrile, generalized, myoclonic and combination of afebrile and febrile seizures are described in these cases. The only anomaly of chromosome 14 with a striking association with epilepsy is the ring 14. Numerous cases are reported, some of which are familial (Singh *et al.,* 2002). Recently reported is a syndrome due to 15q13.3 microdeletion associated with mental retardation, developmental delay and seizures. In these patients, seizures were of various types: myoclonic seizures, absence seizures, tonic-clonic seizures, intractable epilepsy (Sharp *et al.,* 2008). Epilepsy has been an uncommon manifestation associated with 22q11 deletion. It is present in less than 5% of the patients (Roubertie *et al*., 2001)

In the present study numerical chromosome aberrations recorded included trisomy in 13 and 21st chromosomes in a mosaic manner along with structural chromosome aberrations in both groups of patients. In trisomy 13 or Patau syndrome, seizures are reported rarely even if a variety of developmental abnormalities of the brain are present: holoprosencephaly (60-80% of cases), cerebellar dysplastic changes, olfactory aplasia, hippocampal hypoplasia and callosal agenesis. Epilepsy occurs in 8% of individuals with Down syndrome (DS). Age of seizure onset is bimodal: 40% occurs before 1 year of age and 40% occur in the third decade of life (Roizen and Patterson, 2003). The increased seizure susceptibility has been attributed to inherent structural anomalies of the brain such as fewer inhibitory interneurons, decreased neuronal density, abnormal neuronal lamination, persistence of dendrites with foetal morphology or primitive synaptic profiles (Stafstrom *et al*., 1991).

In conclusion, this preliminary study provides valuable information on different types of aberrant karyotypes in epileptic patients of different young age groups in the population studied. Further studies with a large sample size and better classification are needed to delineate the clinical features of epileptic seizures and to understand the mechanisms of epilepsy associated with chromosomal abnormalities.

Acknowledgement

We wish to thank all the faculties of the Coimbatore Medical College Hospital, Coimbatore, TN, India.

Conflict of Interests

No funds were received for this study. Authors do not have any conflicting interests.

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International Journal of
Life Sciences
International Peer Reviewed Open Access Refereed Journal

Special Issue A 11: January 2018
UGC Approved Journal No 48951

Original Article Open Access

Cytogenetic and micronuclei assessment of smokeless tobacco users: A case-control study

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Manuscript details:

Available online on
http://www.ijlsci.in

ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

Editor: Dr. Arvind Chavhan

Cite this article as:
Chandirasekar R, Uthayakumar V, Anitha E, Mekala S, Mathivanan S, Raghadevi M (2018 Cytogenetic and micronuclei assessment of smokeless tobacco users: A case-control study, *Int. J. of. Life Sciences*, Special Issue, A11: 6-10.

ABSTRACT

Tobacco use is the world's leading cause of death, it is estimated that around 4.9 million deaths occur annually due to tobacco, and it is expected to rise to about 10 million by 2030. The use of smokeless tobacco in most of its forms possesses a substantial oral cancer risk at least in the developing countries where they are closely linked to socio cultural conditions. The primary aim of the study was to evaluate the cytogenetic and genotoxic changes in smokeless tobacco users. About 156 smokeless tobacco users have been enrolled and categorized into two groups on the basis of duration of their habit and equal number controls have been enrolled with age and sex matched non-tobacco users. We observed alteration in the incidence of chromosomal anomaly and micronuclei frequencies among the exposed and controls subjects by using the peripheral blood cells and buccal cells. The smokeless tobacco users exhibited varied levels of damages than non-users and predominantly in group II subjects with the higher duration of habit. In conclusion, use of smokeless tobacco for a longer duration caused more damages in comparison short term use and controls and awareness about the harmful effects of tobacco use is very low in rural populations.

Keywords: Tobacco, Genetic, Chromosome aberration, Micronuclei

INTRODUCTION

Tobacco use is a risk factor for many non-communicable diseases and it is an important cause of avoidable death. It is estimated that one billion people die due to usage of different forms of tobacco in the 21st century (WHO 2011). Today, around the world, tobacco is one of the most widely distributed and commonly used addictive substance (Makwana *et al.,* 2007). Globally, around five million deaths every year are attributable to direct tobacco use, which is the largest preventable cause of death (WHO 2012). There are more than one billion smokers worldwide with nearly 80% of them living in low and middle income countries (WHO 2011). In

India, among people aged 30 years and over, the mortality due to tobacco use is 206 per 100000 in men and 13 per 100000 in women with proportion of deaths attributable to tobacco reaching 12% for men and 1% for women (WHO 2012). In India tobacco is being used in smoking as well as smokeless forms (Rani *et al.,* 2003). The genotoxic effect of smokeless tobacco should be considered in addition to other known hazards for assessing health risks (Sudha *et al.,* 2009). Epidemiological data have shown a correlation between the use of smokeless tobacco (SLT) products, premalignant lesions of the oral cavity, and incidence of oral cancer (Rodu *et al.,* 2004).

SLT use is genotoxic and may affect DNA repair pathways (Ishikawa *et al*., 2005). Genotoxic effects in lymphocytes from smokers are most likely caused by cigarette smoke constituents, providing scientific evidence to encourage national campaigns to prevent tobacco consumption (Monica *et al*., 2004). Tobacco smoke induces an array of genetic aberrations including gene mutations, chromosome aberrations (CAs), sister chromatid exchanges and DNA strand breaks (DeMarini, 2004). One of the best techniques for studying the effects of environmental factors on genetic stability in human cells is the micronucleus (MN) test (Nersesyan *et al.,* 2002). MN may be products of early events in carcinogenesis, especially in the oral cavity, which is directly exposed to carcinogens / mutagens present in tobacco (Ramirez and Saldanha, 2002). The aim of this investigation was to analyze the effect of SLT use on the frequencies of chromosomal aberrations and micronucleus in peripheral blood lymphocytes and buccal epithelial cells respectively.

MATERIALS AND METHODS

A total of 156 smokeless tobacco users and an equivalent number of normal controls (156) were recruited for the present study. The subjects recruited for the present study belonged to hilly areas (mostly Tribals) of Western Ghats of Tamil Nadu such as Bargur, Tamaraikarai and Devarmalai of Erode district. The study was approved by the Medical Review Committee, Dhanvantri College of Nursing, Tiruchengode, Namakkal, Tamil Nadu. The subjects were informed about the study and consent was obtained from them. A standard questionnaire was used to collect information regarding the duration of tobacco use, form of tobacco use (smokeless & smoke), their health status, occupation etc. The recruited SLT users were categorised into two groups (Group I: Low duration of use and Group II: Longer duration of use). Of the 156 SLT users 52 were males and 104 were females. An equal numbers of age and sex matched non tobacco users served as controls. By a skilled medical nurse about 2–3 mL of venous blood was drawn from the SLT users and controls into a heparin-coated vacutainer to analyze chromosome aberrations and micronuclei frequency. The buccal epithelial cells were also collected from SLT users and controls in 0.9% saline solution to analyze micronuclei frequency.

Chromosome aberration analysis

Lymphocytes cultures were initiated following a standard procedure (Hoyos *et al.,* 1996). A volume of 0.5 ml blood was added to 4.5 mL RPMI 1640 medium supplemented with 15% FBS, 2 mM L-glutamine, 100U/mL penicillin and 100µg/mL streptomycin and 0.2 mL of PHA. The whole culture was incubated at 37ºC for 72 hrs and after 71 hrs, cultures were treated with 0.01 mg/ml colcemid to arrest the cells at mitotic stage. Lymphocytes were harvested after 72 hrs by centrifuging the cells at 800-1000 rpm, thereafter added pre-warmed (37ºC) hypotonic solution (KCl 0.075 M) and left undisturbed for 20 mins. The cells were fixed in 3:1 ratio of methanol: acetic acid. Slides were prepared and cautiously dried on a hot plate (56ºC, 2 min). Later, slides were stained using Giemsa stain. For the chromosome aberration analysis, 50 well spread metaphase spreads were analyzed for each subject under oil immersion lens of Leica light microscope (100X) and well spread metaphases were photographed.

Micronucleus in peripheral lymphocytes

Cytokinesis blocked micronucleus (CBMN) assay was carried out following the procedure described by Fenech and Morley (1986). In brief, Whole blood (0.5 ml) was added to 4.5 mL of RPMI-1640 medium (Hyclone, USA) supplemented with 20% fetal bovine serum (ThermoFischer Scientific, India), 2mM L-Glutamine (ThermoFischer Scientific, India), and 0.2mL of phytohemagglutinin (ThermoFischer Scientific, India). The mixture was incubated at 37°C for 72 hours. Cytokinesis was blocked by the addition of cytochalasin B (Sigma, India) at a final concentration of 6 µg/ml 44 h after stimulation with phytohemagglutinin. After 72 h of incubation, cells were harvested by centrifugation, given 1 min hypotonic treatment (0.075 M KCl) and fixed in fresh fixative solution (methanol : acetic acid, 3 : 1). This

fixation step was repeated twice after 20 min storage at 4°C and eventually, stained in Giemsa stain. About 500 cells from each SLT user were analysed to estimate micronucleus frequency.

Micronucleus analysis in buccal epithelial cells

After rinsing the mouth with tap water, exfoliated buccal mucosa cells were collected by scraping the right/left cheek mucosa with a wooden spatula. Thus collected cells were transferred to a tube containing saline solution (0.9% NaCl). The cells were centrifuged (800 rpm) for 5 min, fixed in 3:1 methanol/acetic acid, and dropped onto a pre-cleaned slide. Later, the air dried slides were stained in Feulgen plus fast green. The identification of micronuclei was based on the criteria proposed by Sarto *et al*. (1987). About 1,000 cells were screened to estimate the frequency of micronuclei in each subject.

Statistical analysis

Statistical analysis was carried out using the statistical software SPSS Version 16. Analysis of variance (one way-ANOVA) was used to evaluate the frequency of chromosome aberration and micronuclei between SLT users and controls. $P<0.05$ was used as the criterion of significance.

RESULTS

Table 1 show the mean age of SLT users and controls and mean duration of SLT usage among group I and group II SLT users. The SLT users were further categorised based on their age into group I (<35 Years) and group II (>35 Years). Of these two groups, group II harboured more SLT users in comparison with group I. The present study also observed that, SLT usage was highly prevalent among females (66.7%) than males (33.4%) in this population.

Chromosome aberrations such as gaps and breaks were observed in SLT users. Elevated levels of chromosome aberrations (2.65±1.29) were found in group II SLT users in comparison with controls (0.65±0.71). Of the two groups of SLT users chromosome aberrations was found to be higher in group II SLT users (Table 2).

Table1. Demographic details of age and year of exposure in smokeless tobacco users.

Particulars	SLT users Mean ± SD	Control subjects Mean ±SD
Total samples	156	156
Male	52(33.33%)	52(33.33%)
Female	104(66.66%)	104(66.66%)
Age		
Group I (age below 35)	64(34.28±5.56)	64(34.76 ±5.18)
Group II (age above 35)	92(57.56±8.65)	92(53.54±6.68)
Year of exposure		
Group I	64(9.14±3.13)	-
Group II	92(18.57±8.56)	-

SD= standard deviation

Table 2. Frequencies of chromosome aberrations in smokeless tobacco users and controls

Subjects	Number of samples	Chromosome aberrations
SLT users		
Group I	64	1.90±1.17
Group II	92	2.65±1.29*
Controls		
Group I		
Group II	64	0.65±0.71
	92	0.92±0.77

*significantly elevated compared to controls subjects

Table 3. Frequencies of Micronuclei Frequency in smokeless tobacco users and non users

Subjects	Micronuclei Frequency (blood cells/500)	Micronuclei Frequency (buccal cells/1000)
SLT users		
Group I (64)	1.56±1.12*	2.21±1.56#
Group II (92)	2.95±1.61**	3.10±1.46##
Controls		
Group I (64)	0.64±0.60	0.95±0.80
Group II (92)	1.01±0.88	1.15±1.07

*, # significantly elevated compared to controls subjects

**, ## Significantly elevated compared to controls and group I experimental subject

A significantly elevated level of micronuclei frequency was observed in the blood lymphocytes of group II SLT users (2.95±1.61) when compared to the group I SLT users (1.56±1.12) and controls (p<0.05). Similarly, buccal epithelial cells of group II SLT users had significantly increased number of micronuclei (3.10±1.46) than group I SLT users and controls (p<0.05, Table3).

DISCUSSION

Epidemiological studies related to tobacco use are very important to predict cancer risk and mortality and morbidity associated with tobacco related cancer (ICRC, 2003). In South Asia over 250 million people are using SLT products, about 17% of total population in South East Asia use oral tobacco; of which 95% belong to India (82%) and Bangladesh (13%; WHO, 2004). Smoking is responsible for a substantial number of human health problems worldwide (Weir *et al*., 2003). Tobacco smoke induces an array of genetic aberrations, including gene mutations, chromosome aberrations (CAs), micronuclei (MN), sister chromatid exchanges (SCEs), DNA strand breaks, and oxidative DNA adduct in various models (DeMarini, 2004).

CAs and MNs are excellent biomarkers for predicting cancer risk in individuals who are habitual tobacco users. In the present study, CAs like gaps and breaks were observed in elevated levels in group II SLT users than group I and controls. Chromosomal damage has been shown to increase progressively with age and few studies had identified smoking habit as a significant factor that induces alterations in the genetic material (Moacir *et al*., 2010). Jagetia *et al*. (2001) reported that, the chemical carcinogens present in tobacco cause structural alterations in the DNA of target cells, leading to genomic instability in the form of chromosomal abnormalities. Furthermore, Sierra-Torres *et al*. (2004) observed a significantly higher frequency of CAs among smokers compared to non-smokers. The CAs observed in the present study are consistent with a previous study in which CA frequency was significantly increased in smokers in comparison with non-smokers (Tawn and Whitehouse, 2001).

In the present study, the frequency of MN in blood lymphocytes and buccal epithelial cells were found to be higher in group II SLT users than group I SLT users (p<0.05) and controls. Our results are in agreement with a previous study which reported increased MN frequency in smokers (Haveric *et al.,* 2010). In addition to this, Kamboj and Mahajan reported that, even abnormal oral habits significantly increases the frequency of micronuclei (Kamboj and Mahajan, 2007). An another possible reason for the higher micronuclei frequency in group II SLT users may be the age of subjects. Orta and Gunebakan (2012) reported that, micronuclei frequency increases with age of the subjects.

The study also observed that, literacy level of people including the SLT users of the present study inhabiting in these places is very low and also they are very less aware or unaware of the potential health risks associated with tobacco use. Immediate measures need to be taken to educate or create awareness about the ill effects of SLT usage that is extensive among all age groups in this tribal population.

Acknowledgements

We sincerely acknowledge the numerous participants for making this work possible. This study has been carried out by using the funds received from Department of Science and Technology - Science and Engineering Research Board (DST-SERB) (DST/SERB/EEQ/2016/000781).

Conflicts of interest: The authors stated that no conflicts of interest.

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International Journal of
Life Sciences
International Peer Reviewed Open Access Refereed Journal

Special Issue A 11: January 2018
UGC Approved Journal No 48951

Original Article | Open Access

Isolation, identification and characterization of endophytic bacteria- *Azospirillum sp*. and *Pseudomonas sp*. from Brinjal (*Solanum melongena* l.)

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Manuscript details:

Available online on
http://www.ijlsci.in

ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

Editor: Dr. Arvind Chavhan

Cite this article as:
Sivagamasundari U and Gandhi A (2018) Isolation, identification and characterization of endophytic bacteria- *Azospirillum sp*. and *Pseudomonas sp*. from Brinjal (*Solanum melongena* l.), *Int. J. of. Life Sciences*, Special Issue, A11: 11-16.

ABSTRACT

The aim of the present study was to isolate, identify and characterize the bacterial endophytes that reside inside the plant tissue of brinjal as native. Totally twenty isolates (*Azospirillum sp*. and *Pseudomonas sp*.) were isolated from brinjal from three different localities namely, Annamalai university, Putthur, Tamilnadu and Karaikal, Pondicherry, India. All the bacterial isolates were evaluated for its biochemical characterization, IAA production and nitrogen fixation. The results showed that, among the isolates of *Azospirillum sp*., isolate number ABRK 10 and in concerned with Pseudomonas sp., isolate number PBRU3 fix high nitrogen, hence they were selected as efficient strains and molecularly characterized by sequencing their 16srDNA. Sequencing results confirmed that their sequence are native to *Azospirillum brasilense* and *Pseudomonas fluorescens* respectively. This study indicates that there is huge number of endophytic microbes occupy a relatively privileged niche within plant and usually contribute to plant health. From this isolation, it was clear that, bacterial population occupy variety of ecological habitat as unique inside their own host plant as species specific to face their own nutrition, competition and contribute plant health and defence.

Keywords: Endophytes, brinjal, *Azospirillum brasilense, Pseudomonas fluorescens*.

INTRODUCTION

Plant tissues are non-sterile; spaces within them are inhabited by different species of fungi and bacteria known as “endophytes”. Most of these microorganisms are not pathogenic to the host plant. Moreover the association between the plants and its endophytes is very often mutualistic. Endophytic bacteria had been isolated from many different plants including (pine, yew), fodders (alfalfa, sorghum, clover), vegetables

(carrot, radish, tomatoes, sweet potatoes, lettuce, soya bean), fruits (banana, pineapple, citrus), cereal grains (maize, rice, wheat) and other crops (marigold, sugarcane, coffee) (Rosen Blueth and Maritine-Romero, 2006). Numerous studies have demonstrated that endophytes synthesize bioactive compounds which can, for example stimulate plant growth and increase resistance to plant pathogens. It is believed that some part of all the metabolites detected in plant tissues originate from colonizing bacteria.

Among the diversity of endophytes studied in all crops, vegetables are poorly studied. So the aim of the present research was to study the diversity and widespread application of endophytes as inoculants in agriculture fields - *Azospirillum brasilense* and *Pseudomonas fluorescens* colonizing interior tissues of brinjal as their native host. Brinjal or eggplant (*Solanum melongena* L.) is an important solanaceous crop of sub-tropics and tropics. The name brinjal is popular in Indian subcontinents. The name eggplant has been derived from the shape of the fruit of some varieties, which are white and resemble in shape to chicken eggs. In India, it is one of the most common, popular and principal vegetable crops grown throughout the country except in higher altitudes. It is a versatile crop adapted to different agro - climatic conditions and can be grown throughout the year. It is a perennial but grown commercially as an annual crop. A number of cultivars are being grown in India and consumer preference dependent upon fruit color, size and shape.

Brinjal fruit (unripe) is primarily consumed as cooked vegetable in various ways and dried shoots are used as fuel in rural areas. It is low in calories and fats, contains mostly water, some protein, fiber and carbohydrates. It is a good source of minerals and vitamins and is rich in total water soluble sugars, free reducing sugars, amide proteins among other nutrients. Brinjal is known to have ayurvedic medicinal properties and is good for diabetic patients. It has also been recommended as an excellent remedy for those suffering from liver complaints (Shukla and Naik, 1993)

MATERIALS AND METHODS

Collection of samples

Eggplant variety *Annamalai* was collected from Agriculture field of Annamalainagar, Faculty of Agriculture, Annamalai University, Puthur, Tamilnadu and Karaikal, Pondicherry, India. The plant samples collected from three localities were transported in refrigerated box (4°C) to the laboratory.

Method of isolation

Plant parts such as root, stem and leaves of the samples were initially washed with tap water to remove adhering soil particles and soaked in disinfectant solution of 0.1% mercuric chloride for 2 minutes. Then the samples were thoroughly washed in tap water followed by sterile distilled water for 3-4 times. Then they were toweled with sterile filter paper and weighed 1gm and macerated in mortar and pestle and the extract volume was made to 10 mL by adding with sterile distilled water. From that, 1mL was taken and serially diluted with sterilized distilled water.

Isolation of bacterial colony

About 0.1mL each from the dilutions of 10^{-4}, 10^{-5} and 10^{-6} was transferred to petri-plates individually containing nitrogen free malate medium (Dobereiner,1992) and King's B (Schaad, 1980) medium for the isolation of *Azospirillum sp.* and *Pseudomonas sp.* respectively. The petri plates were incubated at 28ºC for 48hrs. Meanwhile 0.5mL of the extracts was transferred to a 5mL sterile semi solid NFB medium and incubated to observe the sub-surface pellicle formation of *Azospirillum sp*. The sub-surface pellicle with change of colour of the medium into blue was further streaked on NFB solid medium to get individual colony of *Azospirillum sp*. The individual colonies from NFB and King's B medium exhibited yellow fluorescent colour and were transferred to slants containing the respective medium. This was incubated at 28ºC for 48 hrs, after that maintained at 4ºC in refrigerator for further study.

Purification of *Azospirillum sp.* and *Pseudomonas sp.*

The isolates of *Azospirillum sp*. were further confirmed by streaking on to potato infusion agar (BMS) plates and incubated at 32ºC for 7days. Individual colonies showing scarlet with typical pink colour often wrinkled colonies were transferred to nutrient agar slants for further study. The petriplates containing King's B medium for *Pseudomonas fluorescens* were examined after incubation under ultraviolet light at 360µm for the confirmation of colonies exhibiting fluorescence.

Characterization of *Azospirillum sp.* and *pseudomonas sp.*

Microscopic observation of the wet mounts of the 72hrs old cultures was carried out for their shape and motility.

Further the isolates were subjected to various biochemical tests for their characterization. The representative isolates of *Azospirillum sp*. and *Pseudomonas sp*. were identified by preliminary identification tests (John Holt *et al.,* 1994) like the Gram Stain, Catalse test, Oxidase test, Spore formation, Carbohydrate fermentation test, Motility test (Hanging drop method) Biotin requirement (Allen, 1953) Nitrate and Nitrite reductase (Yordi and Ruoff, 1981).

Nitrogen Fixation and IAA production

The nitrogen fixation of the isolates was determined by Microkjeldahl Assay described by Humphris, 1956 and IAA production (Gorden and Paleg, 1994).

Molecular characterization and Phylogenetic analysis of endophytic bacterial isolates by 16srRNA sequencing

Isolation of DNA

Total genomic DNA was extracted by standard method. All the isolates were grown at 30°C for 5 days in shake flasks containing 100 mL of NFB and King's B liquid medium for isolates of AORU5 and PBRU3 respectively. Pellet was obtained by centrifugation and washed twice with distilled water. Approximately 200 mg of pellet was used for the genomic DNA extraction. The pellet was suspended in 500 µL of the lysis solution [100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 250 mM NaCl and 2% SDS]. Lysozyme was added to obtain a final concentration of 1 mg/mL and then incubated at 37°C for 60 min. After the addition of 10 µl of proteinase K (10 mg/mL), the mixture was incubated at 65°C for 30 min. The solution was chilled on ice and extracted with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1). The organic extraction was repeated, and the supernatant was taken and 4M Ammonium acetate and two volumes of isopropanol were added. Total genomic DNA was precipitated by centrifugation for 10 min at room temperature. The pellet was washed with 70% ethanol, dissolved in Tris EDTA buffer (pH 8.0) and stored at -20°C.

16SrRNA Sequence

Amplification of 16SrRNA gene sequence was achieved by using 27F (forward primer) and 1492R (reverse primer). PCR setup was prepared to 25µl containing 100ng of template DNA, 2 mM $MgCl_2$, 5µm of primers, 2.5 µl of 10X assay buffer which includes (10 mM Tris (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% Gelatin), 10 mM each of dNTPs and 5units/µl of Taq DNA Polymerase. Initial denaturation at 94ºC for 5 min, 35 cycles of 1 min at 94ºC, 1 min at 45ºC and 1 min at 72ºC, followed by a final 10 min extension at 72ºC.

The amplified products of approximately 1,461 bp of AORU5 and 1.341 bp of PBRU3 were sequenced by pyro sequencing method. Sequence was run in the blast search through National Centre for Biotechnology Information (NCBI) database using the BLAST Programme. Closely related sequences were downloaded and aligned using CLUSTAL X. A phylogenetic tree was constructed using the Neighbour joining method. A phylogenetic and molecular evolutionary analysis was performed using MEGA (Molecular Evolutionary Genetics analysis) version 4.0. Based on the homology 100% similarities were observed with *Azospirillum brasilense* and *Pseudomonas fluorescens* of the isolated AORU5 and PBRU3 respectively.

RESULTS

A total of twenty eight isolates were isolated from brinjal. Of the 28 isolated, 15 were *Azospirillum sp.* and 13 were *Pseudomonas sp.* Eight *Azospirillum sp.* were isolated from the plant sample collected from Annamalainagar, which includes five from root, two from stem and one from leaf. From plant sample of Karaikal, two from root, two from stem and one isolate from leaf were isolated. From the plants collected from Puthur, two were isolated from root.

Concerned with *Pseudomonas sp*. six were isolated from plants collected in Annamalainagar. Of which three were isolated from root, two from stem and only one from leaf. From the plants collected at Karaikal, two were isolated from root, each one from stem and one from leaf. From Puthur plant sample, two from root and one from stem were isolated.

The maximum percentage (3.05) of nitrogen fixation recorded with the ABRK10 and minimum of 1.14% recorded with ABRU2 of isolates of *Azospirillum*. The IAA production ranged from 0.18 to 1.60 µg. In concerned with isolates of *Pseudomonas,* the nitrogen fixation was observed with only two isolates (0.81% and 1.02%). But all the isolates produced IAA ranged from 0.05 to 1.80 µg.

Table: 1 List of bacterial endophytes isolated from different tissues and locality of Brinjal.

S. NO.	BACTERIAL ISOLATES	BACTERIAL NAME	ISOLATED FROM	LOCALITY
1.	ABRU1	*Azospirillum sp.*	Root	Annamalainagar
2.	ABRU2	*Azospirillum sp.*	Root	Annamalainagar
3.	ABRU3	*Azospirillum sp.*	Root	Annamalainagar
4.	ABRU4	*Azospirillum sp.*	Root	Annamalainagar
5.	ABRU5	*Azospirillum sp.*	Root	Annamalainagar
6.	ABSU6	*Azospirillum sp.*	Stem	Annamalainagar
7.	ABSU7	*Azospirillum sp.*	Stem	Annamalainagar
8.	ABLU8	*Azospirillum sp.*	Leaf	Annamalainagar
9	ABRK9	*Azospirillum sp.*	Root	Karaikal
10.	ABRK10	*Azospirillum sp.*	Root	Karaikal
11.	ABRK11	*Azospirillum sp.*	Stem	Karaikal
12.	ABSK12	*Azospirillum sp.*	Stem	Karaikal
13.	ABLK13	*Azospirillum sp.*	Leaf	Karaikal
14.	ABRP14	*Azospirillum sp.*	Root	Putthur
15.	ABSP15	*Azospirillum sp.*	Root	Putthur
16.	PBRU1	*Pseudomonas sp.*	Root	Annamalainagar
17.	PBRU2	*Pseudomonas sp.*	Root	Annamalainagar
18.	PBRU3	*Pseudomonas sp.*	Root	Annamalainagar
19.	PBSU4	*Pseudomonas sp.*	Stem	Annamalainagar
20.	PBSU5	*Pseudomonas sp.*	Stem	Annamalainagar
21.	PBLU6	*Pseudomonas sp.*	Leaf	Annamalainagar
22.	PBRK7	*Pseudomonas sp.*	Root	Karaikal
23.	PBRK8	*Pseudomonas sp.*	Root	Karaikal
24.	PBSK9	*Pseudomonas sp.*	Stem	Karaikal
25.	PBLK10	*Pseudomonas sp.*	leaf	Karaikal
26.	PBRP11	*Pseudomonas sp.*	Root	Putthur
27.	PBRP12	*Pseudomonas sp.*	Root	Putthur
28.	PBSP13	*Pseudomonas sp.*	Stem	Putthur

Table 2: Nitrogen fixation and IAA production of obtained isolates of *Azospirillum sp.* from brinjal.

ISOLATE NUMBER	N FIXATION %	IAA PRODUCTION ($\mu g/ML^{-1}$)
ABRU1	2.21±0.055	1.20±0.025
ABRU2	1.14±0.041	1.60±0.035
ABRU3	2.05±0.026	0.45±0.035
ABRU4	1.52±0.040	1.20±0.612
ABRU5	2.52±0.068	1.16±0.049
ABSU6	1.96 ±0.040	0.33±0.035
ABSU7	2.44±0.045	0.82±0.041
ABLU8	1.52±0.040	0.18±0.007
ABRK9	2.05±0.035	1.09±0.021
ABRK10	3.05±0.056	1.10±0.028
ABRK11	2.02±0.047	1.72±0.049
ABSK12	1.82±0.045	0.19±0.035
ABLK13	1.78±0.045	0.57±0.070
ABRP14	2.18±0.030	1.19±0.014
ABSP15	2.05±0.045	0.65±0.025

Values are ± SD of three samples

Table 3: Nitrogen fixation and IAA production of obtained isolates of *Pseudomonas sp.* from brinjal.

ISOLATE NUMBER	N FIXATION %	IAA PRODUCTION (μGML^{-1})
PBRU1	0.81±0.050	1.28±0.040
PBRU2	-	1.18±0.041
PBRU3	1.02 ±0.052	1.80±0.050
PBSU4	-	0.40±0.042
PBSU5	-	1.24±0.014
PBLU6	-	0.05±0.035
PBRK7	-	1.16 ±0.030
PBRK9	-	0.52 ±0.047
PBSK10	-	0.52 ±0.047
PBLK11	-	0.17±0.02
PBRP12	-	1.18±0.037
PBRP13	-	1.17±0.051
PBSP14	-	1.05 ±5.557

Values are ± SD of three sample

Table 4: Biochemical characteristic of obtained isolates of *Azospirillum sp.* and *Pseudomonas sp.* from brinjal
+ Positive

ISOLATE NUMBER	APG	UTILIZATION OF DIFFERENT CARBON SOURCE				BN	NRA	NIRA	SF	GS	CA	OA	MO
		MAL	SUC										
ABRU1	+	-	+	+	-	+	+	+	-	-	-	+	+
ABRU2	-	+	+	+	-	+	+	+	-	-	+	+	+
ABRU3	-	+	+	+	-	-	+	+	-	+	+	+	+
ABRU4	+	+	-	+	+	+	+	+	-	+	-	+	+
ABRU5	-	+	+	+	+	-	+	+	-	-	-	+	+
ABSU6	+	+	-	+	+	+	+	+	-	-	-	+	+
ABSU7	+	-	+	-	+	-	+	+	-	-	+	-	+
ABLU8	+	-	-	-	+	+	+	+	-	+	-	+	+
ABRK9	-	-	+	-	-	+	+	+	-	-	+	+	+
ABRK10	+	+	+	-	-	-	+	+	-	-	+	+	+
ABRK11	+	+	+	+	+	+	+	+	-	-	-	+	+
ABSK12	+	-	+	+	-	-	+	+	-	-	+	+	+
ABLK13	-	+	-	+	-	+	+	+	-	+	-	+	+
ABRP14	+	+	+	+	+	-	+	+	-	-	+	+	+
ABSP15	-	+	-	+	+	+	+	+	-	+	-	+	+
PBRU1	-	+	-	-	-	+	+	-	-	-	-	+	+
PBRU2	-	-	-	-	-	+	-	-	-	-	+	-	+
PBRU3	-	-	-	+	+	-	+	-	-	-	+	+	+
PBSU4	+	-	-	+	+	-	-	-	-	-	-	+	+
PBSU5	+	+	+	+	+	-	-	-	-	-	+	-	+
PBLU6	+	+	-	-	+	-	-	-	-	-	+	+	+
PBRK7	+	+	-	-	-	-	-	-	-	-	-	+	+
PBRK8	+	-	-	-	+	-	-	-	-	-	-	-	+
PBSK9	-	+	-	-	-	-	-	-	-	-	+	+	+
PBLK10	+	-	+	+	-	-	-	-	-	-	-	+	+
PBRP11	-	+	+	+	+	-	-	-	-	-	+	+	+
PBRP12	+	+	+	+	+	-	-	-	-	-	-	-	+

APG: ACID PRODUCATION FORM GLUCOSE; **MAL:** MALATE; **SUC:** SUCCINATE; **MAN:** MANNITOAL; **FRU:** FRUCTOSE; **BN**: BIOTIN NEEDS; **NRA:** NITRATE REDUCTASE ACTIVITY; **NIRA:** NITRITE REDUCTASE ACTIVITY; **SF:** SPORE FORMATION, **GS:** GRAM'S STAIN; **CA:** CATALASE ACTIVITY; **OA:** OXIDASE ACTIVITY; **MO:** MOTILITY

On the basis of the results obtained, it could be concluded that brinjal harboring diverse group of bacterial endophytes. However, the benefits of these bacterial endophytes are not yet clearly understood. We hypothesize that isolated bacterial endophytes might be useful to its respective host species. Nonetheless, our research findings could be useful, as a foundation for further research on both in agriculture, particularly vegetable production, as well as its endophytic bacteria.

CONCLUSION

A considerable research effort is required to inoculate endophytic bacteria. In order to guarantee reproducibility, reliable methods of inoculum delivery should be developed. This is especially for the inoculation of trees. It should be noted that the development of successful application technologies would fully depend on improving our understanding of how bacterial endophytes enter and colonize plants. This remark could be applied to all aspects of the ecology of bacterial endophytes and only under those circumstances can the potential use of bacterial endophytes for plant beneficial purposes be fully evaluated.

Acknowledgement
The authors wish to thank UGC-SAP, BSR – Fellowship, New Delhi for financial support for this research work.

Conflicts of interest: The authors stated that no conflicts of interest.

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International Journal of
Life Sciences
International Peer Reviewed Open Access Refereed Journal

Special Issue A 11: January 2018
UGC Approved Journal No 48951

Original Article | Open Access

Assessment of hepatoproductive effect of spiny Eel fish Oil against alcohol induced liver marker enzymes and genotoxity in Albino Rats

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Manuscript details:

Available online on
http://www.ijlsci.in

ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

Editor: Dr. Arvind Chavhan

Cite this article as:
Ragadevi M, Chitra E, Jayasurya K, Sakthivel M, Uthayakumar V, Chandirasekar R, Sreedevi PR, Ramasubramanian V, and Sivasankar V (2018) Assessment of Hepatoproductive Effect of Spiny Eel fish Oil against Alcohol Induced Liver Marker Enzymes and Geno-toxity in Albino Rats, *Int. J. of. Life Sciences*, Special Issue, A11: 17-24.

ABSTRACT

In the present study hepatoprotective activity of fish oil against alcohol induced hepatic damage in albino rats was evaluated. Hepatic injury was induced by administering 3 mL of 40% alcohol orally. The levels of liver marker enzymes such as ALT, AST, ACP, ALP, LDH, SGPT, SGOT and GGT significantly increased ($P<0.05$) in alcohol induced hepatic injury group when compare to control. But, when the rats are administered with the combination of Eel fish and Cod liver oil mixed standard diet, the levels of liver marker enzymes showed a better reduction than in rats administered with standard diet (control) and Eel fish oil standard diet. The level of DNA damage was assessed by Comet assay and the Mean Tail Length and Mean Tail Movement was found to be significantly lower ($P<0.05$) in rats administered with alcohol plus Eel and Cod liver oil mixed standard diet in comparison with control (standard diet) and Eel fish oil mixed standard diet groups. The number of micronucleated hepatocytes was significantly reduced in rats administered with Eel and Cod liver oil mixed standard diet (0.3616±0.04633) than in standard diet (1.4426±0.30871). On the whole, our study concludes that, oral administration of Eel and Cod liver oil reversed the alcohol induced hepatic injury and thus it can be used as a hepatoprotective agent.

Key words: Eel fish oil, Liver enzymes, Comet assay, Alcohol, Hepatoproductive.

INTRODUCTION

Liver diseases are mainly caused by toxic chemicals, excessive consumption of alcohol, liver infections and autoimmune disorders. Most of the hepatotoxic chemicals damage liver cells mainly by inducing lipid

peroxidation and other oxidative damage. Alcohol induced liver injury (ALI) and disease (ALD) is the major health problems affecting a broad patient population of different gender, race, and social backgrounds (Barrio *et al.,* 2004). In spite of tremendous strides in modern medicine, there are hardly any drugs that stimulate liver function, offer protection to the liver from damage or help regeneration employed in traditional system of medicine for liver affections of hepatic cells (Clinard and Ouazrir, 2002). However, there is no satisfactory therapy for alcoholic liver disease at present.

Long term alcohol consumption prolongs the inflammatory process leading to excessive production of free radicals, which can destroy healthy liver tissue (Nanji, 1994). Determination of the activity of hepatic enzymes released into the blood by the damaged liver is one of the most useful tools in the study of hepatotoxicity. In animals and man, xenobiotic metabolizing enzyme systems are present in most if not all tissues, with the highest concentration found in liver (Ennulat *et al.,* 2010). Expression of these enzymes is influenced by a range of factors including diet, sex, age, environmental exposures, and most importantly, endobiotics and xenobiotic, including drugs and chemicals. Although drug-metabolizing enzyme (DME) systems in the liver, can be rapidly and reversibly up regulated in response to endogenous or exogenous stimuli, a process known as enzyme induction. Increased alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT) activities in liver parenchyma have been described in association with drug-induced CYP450 induction in the rat, the dog, and the human (Xu *et al*., 2005). The serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) are synthesized in the liver, heart and skeletal muscles. Elevation of these enzymes indicates liver, heart, muscle or brain injury (Calbreth, 1992).

Fish fatty acid s is considered as a strong antioxidant and its role as an anticancer agent has been extensively confirmed in most of the human malignancies (Sheikh *et al.,* 2010). Its role in enhancement cytotoxity of anticancer drugs to tumor cells and protection of normal cells was previously reported (Pardini, 2006). Furthermore, the anti inflammatory potential of long chain Omega-3 fatty acids in many chronic diseases has been suggested (Wall *et al.,* 2010). The role of Omega-3 fatty acids in inhibiting proliferation, inducing apoptosis and promoting differentiation in many cancers have been studied (Sun *et al.,* 2009) and relevant finding indicate that fish oil act synergistically with certain chemotherapeutic agents (Wendel and Hellar, 2009). The present study aimed to find the hepatoprotective efficacy of Eel fish and Cod liver oil mixed standard diet in Wistar rats by analyzing liver marker enzymes and the markers of genotoxic effects.

MATERIALS AND METHODS

Study Approval

The study was approved by the Institutional Animals Ethics Committee (722/02/a /CPCSEA-dt 04.12.2006) at Bharathiar University, Coimbatore, Tamil Nadu, India. The Wister strain rats were maintained as per the recommendation of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines and regulations.

Experimental Protocol

Animals were divided into five Experimental groups. **Group I-** (+) Control (standard diet), **Group-II:** (-) Control (alcohol+diet), Group**-III:** Cod liver Oil 5ml/kg/ 3times/day (alcohol+diet). **Group-IV:** 40% Alcohol and Standard Diet mixed with Spiny Eel fish Oil (5ml/kg). **Group-V**: 40% Alcohol and Standard Diet mixed with Spiny Eel fish Oil and Cod Liver Oil.

Liver marker enzymes activity

Experimental animals were sacrificed by cervical dislocation and a part of the liver was washed with ice cold tris-buffered saline, blotted dry and 10% homogenate was prepared using tris buffered saline (pH 7.4). The liver homogenate was centrifuged at 2000 rpm for 10 minutes and the supernatant was used for the experiment. Enzymes like AST, ALT, LDH, GOT and GPT were determined by the method of Reitman and Frankels, 1957. ALP and ACP activity was assayed by the method of Kings Armstrong, 1934. Gamma glutamyl transferase enzyme activity was analysed by the method of Rosalki *et al*., 1970.

Comet assay

The alkaline Comet assay was carried out according to the method described by Singh *et al.,* 1988. At the end of treatment, hepatic cells from liver was removed from rats of experimental groups and control group and thus collected cells washed thrice in Phosphate Buffered Solution (PBS**)** and applied on to a microscope slides with agarose in PBS. The slides were then incubated for 20

min in ice-cold electrophoresis solution, followed by electrophoresis. Measuring the lengths of DNA migration (Comet tail) in these cells was carried out directly by fixing an ocular micrometer in one of eyepieces of the microscope. About 60-100 comets per point were scored.

Micronucleus in Peripheral lymphocytes (Fenech and Morley, 1986)

Hepatic cells were collected from liver at the end of treatment by sacrificing the rats of experimental groups and control groups. Immediately after, live cells were added to 5 ml RPMI-1640 medium with 25% foetal bovine serum, 1% L-Glutamine and 2% phytohaemagg-lutinin. After 72 hour of incubation, cells were harvested by centrifugation. Before dropping cell suspension over the slide, slides were wiped with a small amount of cold fixative and the cell suspension dropped and dried over a hot plate maintained at 40°C. After that, cells were stained with Giemsa and gently washed with distilled water to remove the excess stain. Then, the slides were scanned under a high power microscope to score micronucleated lymphocytes.

Statistical analysis

All the statistical analysis was performed using SPSS 16.0 for windows. One-way ANOVA was used to analyze and compare the results of liver enzymes, DNA tail length and movement and number of micronucleated hepatocytes between different groups of rats. $P<0.05$ was considered as significant.

RESULTS

Liver Marker Enzymes

During the short-term (30 days treatments), the levels of liver marker enzymes such as ALT, AST, ACP and ALP were found to be significantly lower ($P<0.05$) in group V (Alcohol+ Eel fish and Cod liver oil mixed standard diet), group IV (Alcohol+ Eel fish oil mixed standard diet), group III (Alcohol+ Cod liver oil mixed standard diet) in comparison with Group II (Alcohol+standard diet). During the long term (90 days treatment), ALT, AST, ACP and ALP levels were found to be significantly lower ($P<0.05$) in group V, IV, III in comparison with group II (Alcohol+standard diet). When the duration of trea-tment was compared, long term treatment was found to be effective than short term treatment (Table 1).

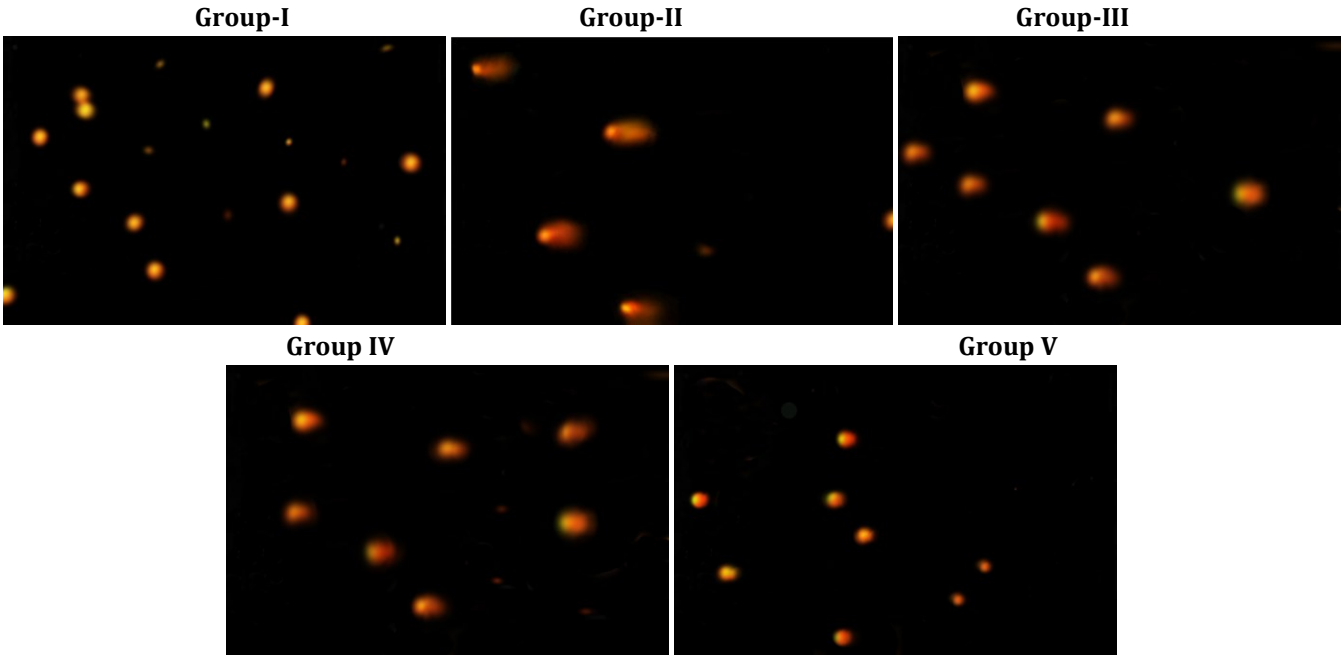

Figure 1 : Photograph of Comet assay in the Control and Experimental Groups rats

Group I – Standard diet; **Group II** – Standard diet+3mL of 40% alcohol; **Group III** - Standard diet+3mL of 40% alcohol+5mL/kg Cod liver oil; **Group IV** - Standard diet+3mL of 40% alcohol+5mL/kg Eel oil; **Group V** - Standard diet+3mL of 40% alcohol+5mL/kg combination of Eel fish oil and Cod liver oil.

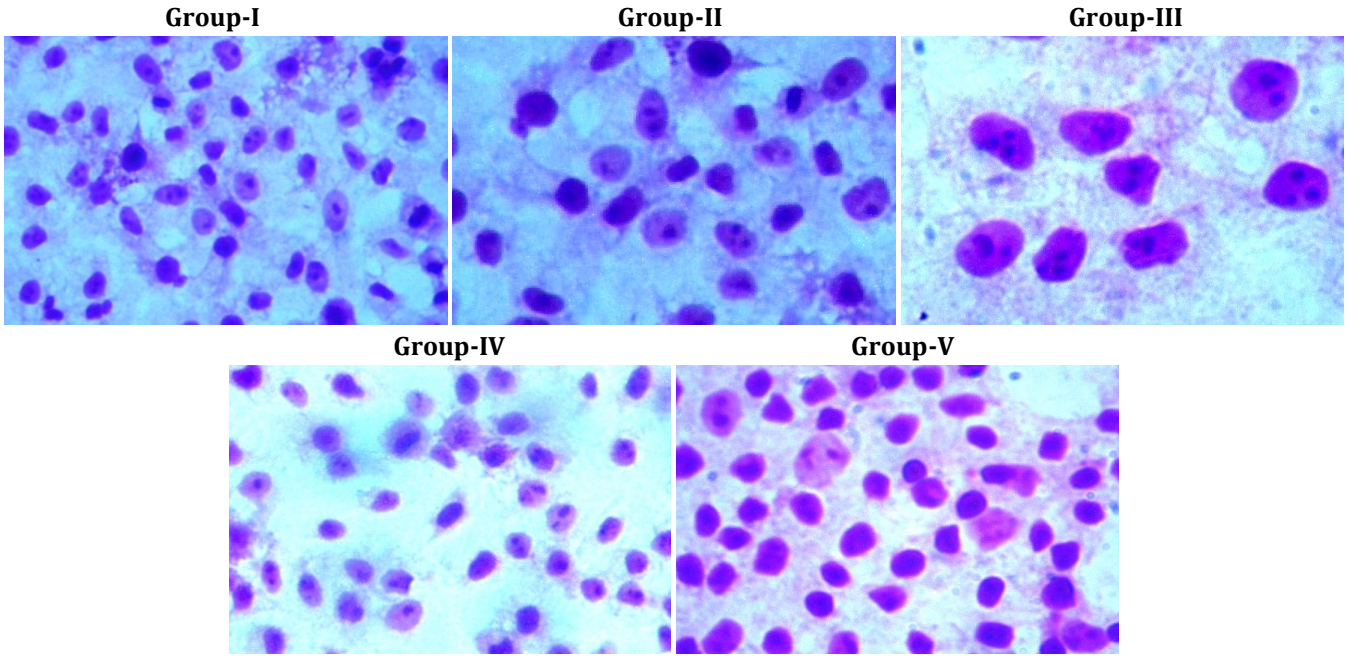

Figure 2: Photograph of Micronuclei in the Control and Experimental Groups rats

Group I – Standard diet; **Group II** – Standard diet+3mL of 40% alcohol; **Group III** - Standard diet+3mL of 40% alcohol+5mL/kg Cod liver oil; **Group IV** - Standard diet+3mL of 40% alcohol+5mL/kg Eel oil; **Group V** - Standard diet+3mL of 40% alcohol+5mL/kg combination of Eel fish oil and Cod liver oil.

Table1: Levels of Alanine transaminase (ALT), Aspartate transaminase (AST), Acid Phosphatase (ACP), Alkaline Phosphatase (ALP) in Liver and Serum of rats

Days	Groups	ALT Serum: Units/ml Liver: micro moles of Pyruvate liberated /min/mg/protein		AST Serum: Units/ml Liver: micro moles of pyruvate liberated /min/mg/protein		ACP Units: micro moles of phenol liberated /min/mg/protein		ALP Units: micro moles of phenol liberated /min/mg/protein	
		Liver	Serum	Liver	Serum	Liver	Serum	Liver	Serum
30 days	I	0.48 ±0.031^{f}	0.71 ±0.010^{g}	0.42 ±0.062^{g}	0.65 ±0.015^{d}	4.34 ±0.026^{i}	5.64 ±0.360^{h}	2.47 ±0.067^{h}	4.23 ±0.502^{g}
	II	0.76 ±0.020^{c}	1.95 ±0.012^{b}	0.98 ±0.038^{b}	1.35 ±0.045^{b}	8.12 ±0.079^{b}	9.86 ±0.045^{b}	7.87 ±0.017^{b}	11.11 ±0.952^{b}
	III	0.67 ±0.020^{d}	1.08 ±0.012^{d,e}	0.62 ±0.081^{c,d}	0.77 ±0.014^{d}	6.72 ±0.020^{e}	7.69 ±0.008^{e}	4.69 ±0.073^{e,f}	7.30 ±0.603^{c}
	IV	0.67 ±0.017^{d}	1.19 ±0.012^{c,d}	0.59 ±0.074^{d,e}	0.73 ±0.046^{d}	6.48±0.086^{f}	7.53 ±0.014^{f}	4.98 ±0.188^{f}	6.90 ±0.080^{c,d}
	V	0.61 ±0.014^{e}	1.02 ±0.120^{e}	0.48 ±0.045^{e,f}	0.69 ±0.014^{d}	6.43 ±0.053^{f}	7.59 ±0.040^{f}	4.36 ±0.065^{f}	7.21 ±0.686^{c}
90 days	I	0.76 ±0.034^{c}	0.87 ±0.320^{f}	0.67 ±0.016^{c,d}	0.71 ±0.010^{d}	5.43 ±0.028^{h}	6.61 ±0.120^{g}	3.20 ±0.524^{g}	6.62 ±0.063^{d}
	II	1.84 ±0.036^{a}	2.91 ±0.140^{a}	2.90 ±0.316^{a}	4.76 ±0.160^{a}	13.55 ±0.070^{a}	12.89 ±0.08^{a}	9.40 ±0.593^{a}	15.19 ±0.035^{a}
	III	0.87±0.022^{b}	1.87 ±0.075^{b}	0.73 ±0.033^{c}	1.23 ±0.012^{b,c}	7.08 ±0.054^{d}	8.94 ±0.029^{c}	4.98 ±0.283^{d,e}	5.98 ±0.091^{e}
	IV	0.91 ±0.054^{b}	1.98 ±0.236^{b}	0.87 ±0.010^{b}	1.19 ±0.000^{b,c}	7.61 ±0.044^{c}	8.76 ±0.030^{d}	5.50 ±0.432^{c,d}	5.88 ±0.091^{e}
	V	0.79 ±0.058^{c}	1.23 ±0.102^{c}	0.98 ±0.014^{b}	1.15 ±0.506^{c}	6.31 ±0.021^{g}	7.66 ±0.075^{e}	5.61 ±0.911^{c}	5.29 ±0.022^{f}

Mean± SD (n=5)

Mean values within the same row sharing the same superscript are not Significant different (P>0.05)

Table 2: Levels of LDH, SGPT, SGOT, GGT in liver and serum of rats

Days	Groups	ALT Serum: Units/ml Liver: micro moles of Pyruvate liberated /min/mg/protein		AST Serum: Units/ml Liver: micro moles of pyruvate liberated /min/mg/protein		ACP Units: micro moles of phenol liberated /min/mg/protein		ALP Units: micro moles of phenol liberated /min/mg/protein	
		Liver	Serum	Liver	Serum	Liver	Serum	Liver	Serum
30 days	I	36.32 ±0.816^{e}	18.76 ±2.440^{e}	65.33 ±0.693^{f}	11.21 ±1.474^{h}	106.25 ±1.500^{g}	57.50 ±2.64^{f}	9.03 ±0.304^{g}	5.26 ±0.315^{d,e}
	II	67.32 ±4.242^{b}	29.76 ±0.778^{b}	68.70 ±2.097^{d}	23.07 ±1.00^{c}	230.99 ±1.818^{b}	76.50 ±1.290^{c}	17.04 ±1.79^{c}	8.34 ±0.509^{b}
	III	42.76 ±3.559^{c}	20.13 ±4.082^{d}	63.72 ±0.920^{g}	12.25 ±0.79^{h}	100.23 ±0.962^{i}	67.50 ±0.577^{e,d}	13.04 ±0.744^{e}	4.08 ±0.681^{f}
	IV	43.98 ±1.632^{c}	18.54 ±3.162^{e}	68.96 ±0.206^{d}	17.27 ±0.426^{g}	108.71 ±1.251^{f}	66.0 ±0.816^{f}	11.71 ±0.335^{f}	4.94 ±0.302^{e}
	V	40.94 ±6.531^{c}	17.61 ±2.82^{e}	62.44 ±0.417^{h}	18.93 ±1.021^{f}	103.49 ±0.571^{h}	58.50 ±1.29^{f}	13.76 ±0.102^{e}	5.31 ±0.252^{d,e}
90 days	I	37.54 ±2.449^{d,e}	24.65 ±5.656^{c}	67.40 ±0.469^{e}	11.77 ±0.272^{h}	121.99 ±1.000^{e}	57.75 ±1.258^{f}	10.86 ±0.84^{f}	7.02 ±0.405^{c}
	II	87.49 ±2.943^{a}	34.88 ±3.265^{a}	178.66 ±0.833^{a}	43.52 ±0.994^{a}	353.06 ±4.375^{a}	126.25 ±1.89^{a}	25.83 ±0.872^{a}	9.24 ±0.602^{a}
	III	43.08 ±3.559^{c}	23.26 ±1.290^{c,d}	72.91 ±0.060^{b}	21.05 ±0.659^{e}	164.12 ±1.36^{e}	69.75 ±3.685^{d}	18.31 ±0.842^{b}	5.66 ±0.021^{d}
	IV	44.65 ±2.828^{c}	25.87 ±1.414^{c}	70.85 ±0.645^{c}	22.0 ±0.816^{d}	167.10 ±0.754^{d}	68.0 ±2.44^{d,e}	15.96 ±0.176^{d}	6.79 ±0.017^{c}
	V	42.78 ±2.160^{c}	22.91 ±1.632^{c,d}	60.46 ±0.646^{i}	26.67 ±0.483^{b}	214.60 ±3.049^{c}	92.0 ±0.816^{b}	18.93 ±0.087^{b}	5.66 ±0.008^{d}

Mean± SD (n=5)

Mean values within the same row sharing the same superscript are not Significant different (P>0.05)

Table 3: Effect of different diet on alcohol induced liver damage

Experimental Groups	Dose (ml/kg)	No. of rats	No. of Mortality	Exposure Periods (Days)	No. of Cells Analyzed	% of DNA Damage	Tail Length (µm)	Mean Tail Movement (%)
Positive Control (+ve)	Standard Diet	6	-	90	100	0^{e}	0^{d}	0^{c}
Negative Control (- ve)	Standard Diet +3ml of 40% Alcohol Thrice a Day Orally	6	2	90	100	40.71 ±3.979^{a} *(0.39716)	63.42 ±14.536^{a} *(01.45126)	22.55 ±8.061^{a} *(0.70544)
Ex1 (Cod Liver Oil)	Standard Diet +3ml of 40% Alcohol+ Cod Liver Oil (5ml/ kg) Thrice a Day Orally Administrated	6	-	90	100	12.26 ±1.306^{b} *(0.13356)	12.94 ±2.734^{c} *(0.27190)	2.07 ±2.384^{b} *(0.25512)
Ex2 (Spiny Eel Oil)	Standard Diet +3ml of 40% Alcohol+ Spiny Eel Oil(5ml/kg) Thrice a Day Orally Administrated	6	-	90	100	4.42 ±0.776^{d} *(0.08011)	11.38 ±2.356^{c} *(0.23753)	0.508 ±0.180^{c} *(0.01812)
Ex3 (Combined Fish oil-Cod + Eel)	Standard Diet +3ml of 40% Alcohol+ Combined Oil (Cod and Eel oil (5ml/kg) Thrice a Day Orally Administrated	6	-	90	100	10.95 ±1.191^{c} *(0.11973)	19.39 ±2.527^{b} *(0.25496)	2.11 ±0.320^{b} *(0.03299)

Mean± SD (n=5)
SE (n=5)

Mean values within the same row sharing the same superscript are not Significant different (P>0.05)

Table 4: Effect of different diet on the number of micronucleated hepatocytes in alcohol induced liver damage

Experimental Groups	Dose (ml/kg)	No. of rats	No. of Mortality	Exposure Periods (Days)	No of Cells Analyzed	MNHEP%/2000 mean Standard Deviation
Positive Control (+ve)	Standard Diet	6	-	90	2000	0.0437 ±0.02208 *(0.00049)
Negative Control (- ve)	Standard Diet +3ml of 40% Alcohol Thrice a Day Orally Administrated	6	2	90	2000	1.4426 ±0.30871 *(0 .00690)
Ex1 (Cod Liver Oil)	Standard Diet +3ml of 40% Alcohol+ Cod Liver Oil(5ml/kg) Thrice a Day Orally Administrated	6	-	90	2000	0.4612 ±0.40060 *(0.00896)
Ex2 (Spiny Eel Oil)	Standard Diet +3ml of 40% Alcohol+ Spiny Eel Oil(5ml/kg) Thrice a Day Orally Administrated	6	-	90	2000	0.2493 ±0.25600 *(0.00572)
Ex3 (Combined Fish oil-Cod + Eel)	Standard Diet +3ml of 40% Alcohol+ Combined Oil (Cod and Eel oil (5ml/kg) Thrice a Day Orally Administrated	6	-	90	2000	0.3616 ±0.04633 *(0.00104)

Mean± SD (n=5)
SE (n=5)
Mean values within the same row sharing the same superscript are not Significant different (P>0.05)

DISCUSSION

In the present study, fish oils decreased the levels of AST and ALT towards the respective control values that were an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by alcohol. A higher level of AST and ALT in the circulation indicates disintegration of cell membrane of liver. The damage provoked by free radicals to macromolecule plays an essential role in the pathophysiological process of atherosclerosis, inflammation, carcinogenesis, aging, drug reaction and toxicity. Alcohol-induced hepatic tissue damage is mediated by acetaldehyde and reactive oxygen species (Zima *et al.,* 2001).

In the present study Cod liver and Eel oil at a dose of 5mL/kg caused a significant decrease in the levels of SGOT and SGPT towards the respective normal range and this is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by ethanol. Our results are in agreement with a previous study in which Omega 3 fatty acids reduced paracetamol and ethanol induced hepatic injury in rats (Meganathan *et al.,* 2011).

Alcohol drinkers accumulate acetaldehyde in the body and consequently suffer from its genotoxic damage (Singh and Khan, 2010). Alcoholics have been found to have a higher frequency of chromosomal aberrations, sister chromatid exchanges and micronuclei in their lymphocytes than non-alcoholics (Maffei *et al.,* 2000), which is in line with the results of our study, with some slight differences.

DNA damage was detected in liver cells of rats following administration of alcohol. The rats administered with Eel fish and Cod liver oil mixed standard diet showed reduced DNA damage than rats administered with standard diet. A possible reason for the observed reduction in DNA damage in rats administered with fish oil mixed diet may be due to its anti-carcinogenic or anti-oxidant properties. Several investigations documented its beneficiary effect against DNA damage. The fish oil contains various biologically important molecules with anti-cancer and anti-oxidant properties. Omega poly unsaturated fatty acids (PUFAs) present in the eel oil inhibit the arachidonic acid pathway. Eicosapentanic acid (EPA) and Docosahexanic acid (DHA) which are abundant in fish oil, suppress colon carcinogenesis in experimental animals (Takahashi *et al.,* 1997). Conjugated linoleic acids are reported to show anti-carcinogenic properties (Narisawa *et al.,* 1991).

On the whole, our study demonstrated that, fish oil mixed diet can reverse the genotoxic effects of ethanol in ethanol induced hepatic injury. Hence, it is suggested

that, fish oil may be an effective dietary supplement in the management of alcohol induced liver damage. However, detailed studies are required to establish the toxicity and protective effect of this fish oil on ethanol-induced liver disorders in humans before it can be recommended for clinical trials.

Acknowledgments

We the authors are grateful to thank the University Grant Commission (UGC) for supporting this research work.

Conflicts of interest: The authors stated that no conflicts of interest.

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International Journal of
Life Sciences
International Peer Reviewed Open Access Refereed Journal

Special Issue A 11: January 2018
UGC Approved Journal No 48951

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Phenol Free and FTA card based Genomic DNA Extraction from Stems and Leaves of *Commiphora wightii* and *C. myrrha*

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Manuscript details:

Available online on
http://www.ijlsci.in

ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

Editor: Dr. Arvind Chavhan

Cite this article as:
Sairkar Pramod Kumar, Sharma Anjana, and Shukla NP (2018) Phenol Free and FTA card based Genomic DNA Extraction from Stems and Leaves of *Commiphora wightii* and *C. myrrha*, *Int. J. of. Life Sciences*, Special Issue, A11: 25-30.

ABSTRACT

"Taxonomically difficult" family Burseraceae are drought-tolerant and leafless for most of the year, contains higher amount of polysaccharides, polyphenols and other secondary metabolites in stems and leaves, which make difficulty in the isolation of genomic DNA. Out of three phenol free CTAB based methods (M-1, M-2 and M-3), the isolated DNA from leaves and stem using method M-2 and M-3 respectively, showed a prominent DNA band and these DNA were also performed good amplification with RAPD primers. Whereas, out of 5 sample application methods on Whatman FTA PlantSaver Card, the sample application of first and finally precipitated DNA of CTAB based M-1 method on FTA cards, showed prominent amplification. Good quality and quantity DNA was obtained as higher concentrations of CTAB, PVP and β-mercaptoethenol treatment was given. The isolated DNA and modified sample application method on the FTA PlantSaver card showed good PCR amplification.

Key worlds: Commiphora, *C. wightii, C. myrrha*, Whatman FTA PlantSaver Card, Genomic DNA, CTAB, PVP, β-mercaptoethenol

INTRODUCTION

In the recent years, herb based medicinal systems like Ayurveda, Unani, Homeopathy and folk medicine systems are widely preferred because allopathic medicines have various side effects (Bakhru 1998). Plant drugs constitute as much as 25% of the total drugs in the USA, while in India, the plant drug contribute 80% of the total drug (Mouli and Rao 2009). Every medicinal plant has their own specific medicinal property due to the presence of higher concentrations of polysaccharides, polyphenols, proteins, and other secondary metabolites of plant leaves which may pose hindrance in the isolation of DNA and creates a problem in genomic

downstream studies (Pandey *et al,* 1996; Haque *et al,* 2009; Sahu *et al,* 2012). One of the major factors affecting the success of genomic studies is the purity of DNA as extraction of quality DNA is a major challenge for molecular biologists dealing with higher plants (Anuradha *et al,* 2013). Various chemical treatments are used for eliminating these compounds (Dabo *et al,* 1993; Haque *et al,* 2009; 2010). Mostly young leaves are used for the isolation of genomic DNA, but other parts of the plant such as stem were also used. Whatman FTA (Flinders Technology for Analysis; Guthrie and Susi 1963) card is widely used in the extraction, long term storage and amplification of DNA from animal tissues, including blood in room temperature, while for the extraction of plant genomic DNA a special FTA Plant Saver card was used (Sairkar *et al,* 2013a; Tsukaya *et al,* 2005).

Commiphora spp. of the family *Burseraceae* is being used as a medicinal plant since ancient times and now rated as an endangered plant species (IUCN 2012). At the time of collection of samples in the year 2010, this species was rated as Data Deficiency in India. They are found in the arid to semiarid regions of the world, including the deserts of India, Pakistan, Africa and Saudi Arabia, while in India, it is found in Rajasthan, Madhya Pradesh, Gujarat, Tamilnadu, Orissa and Karnataka. About 185 species of *Commiphora* were found worldwide, out of them especially *C. wightii* (synonym *C. mukul*) and *C. myrrha* found in India (Kirtikar and Basu 1935; Hocking 1993). *C. wightii* contain a bitter gum known as Guggul (Myrrh) in stems and leaves, which is used in the treatment of hypercholesterolemia and cardiovascular diseases (Deng 2007) and it is also shown to have anti-cancerous activity (Xiao and Singh 2008).

The classification and nomenclature of *Burseraceae*, have been called "taxonomically difficult". The drought-tolerant plants are leafless for most of the year and are thus difficult to identify (Gachathi 1997). *Commiphora* species contains higher amount of polysaccharides, polyphenols and other secondary metabolites in stems and leaves, which make difficulty in the isolation of genomic DNA (Haque *et al,* 2009). With keeping of these problems, DNA of *C. wightii* and *C. myrrha* was isolated from young leaves and young stems through different CTAB based methods. Intended for fast and easy removal of polysaccharides, polyphenols and other secondary metabolites and long term storage of genomic DNA at room temperature, the FTA PlantSaver card was also tried with several sample application methods.

MATERIALS AND METHODS

The samples of two different species of *Commiphora* were collected from Bhopal, Prof. TS Murthy Science Station, Obaidullaganj (Madhya Pradesh), Dr. Panjabrao Deshmukh Agricultural University, Akola (Maharashtra), Anand Agricultural University, Anand (Gujarat), and conserved at MPCST Human Herbal Health Care Garden, Bhopal. All the permission related to this work was taken from MP Council of Science and Technology, Bhopal, Madhya Pradesh and Research Degree Committee, Rani Durgavati University, Jabalpur, Madhya Pradesh.

The genomic DNA was isolated using the young leaves as well as young stem of *Commiphora* spp. through three various CTAB based methods i.e. M-1 method (CTAB method of Vijay *et al,* 2009), M-2 method (CTAB method of Haque *et al,* 2008) and M-3 method (modified method of M-2 method). Methods M-1 and M-2 were used for the isolation of genomic DNA from young leaves of *Commiphora* spp. (*C. wightii* and *C. myrrha*), while genomic DNA from young stem was isolated using the M-2 method and its modified version (M-3). In the method M-3, the entire step till drying the DNA pellet was followed according to standard method of M-2. After drying, the pellet was dissolved in 500 µl high salt TE buffer and an equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 13000 RPM for 10 minutes at room temperature. After centrifugation the aqueous phase was transferred to a fresh tube and 500 µl pre-chilled ethanol was added. The mixture was incubated for 30 minutes at -20ºC and centrifuged at 13000 RPM for 15 minutes at 4ºC. Then the supernatant was decanted carefully and the pellet was washed with 70% ethanol twice, after drying at room temperature pellet was dissolved in 50 µl TE buffer.

Samples were applied on to the Whatman FTA PlantSaver Card from 5 various sample application methods and the FTA card disc was purified for further uses (Whatman 2009; Sairkar *et al,* 2013a). In the first three sample application method, namely, directly press method, homogenized with PBS and homogenized with DNAse, RNAse free water was applied as per the instructions of the manufacturer (Whatman 2009). In the last two methods, the first precipitated DNA and finally precipitated DNA of the M-1 method was dissolved in DNAse, RNAse free water and applied directly on the FTA PlantSaver card. The card was allowed to air dry and stored at room temperature. 2.0

mm disc was washed as per the instructions provided by the manufacturer (Whatman 2009).

The yield of DNA was measured using a NanoDrop UV-Spectrophotometer (ND-1000) at 260 nm using TE buffer as blank. Good quality and quantity obtained DNA was rechecked through 1% agarose gel and amplification by RAPD primers RPI-05 (AM 773770) and RPI-10 (AM 750045) (Sairkar *et al,* 2012; Sairkar *et al,* 2013b).

RESULTS

The obtained DNA isolated from young leaves of *C. wightii* and *C. myrrha* using phenol free CTAB based method M-1 described by Vijay *et al,* (2009) was highly contaminated as well as very low in quality (0.84 to 1.02 ratio of A_{260}/A_{280}) and quantity (99.83 to 132.28 ng/µl) (Table 1). The presence of polysaccharides, polyphenols and other secondary metabolites in the leaves may be co-precipitated with DNA and might be the possible reason for the contamination of the isolated DNA. These unwanted compounds from the *C. wightii* DNA could be eliminated by using higher concentrations of CTAB, PVP and β-mercaptoethenol (Haque *et al,* 2008; Samantaray *et al,* 2009). CTAB binds with polysaccharides, while PVP forms insoluble complexes with lactones and phenolic compounds, whereas β-mercaptoethenol helps in the oxidation of phenolic compound (Kim *et al,* 1997). In the M-2 method, good quality (1.79 to 1.91 ratio of A_{260}/A_{280}) and quantity of DNA (1240.73 to 1670.34 ng/µl) was obtained because it had higher concentrations of CTAB, PVP and β-mercaptoethenol (Table 1). The isolated DNA showed good PCR amplification, therefore it can be further used in for molecular downstream applications (Figure 1).

Commiphora species are drought-tolerant plants and does not carry leaf in most seasons of the year (Gachathi 1997). In case of perennial plants, it is important to isolate the DNA at anytime rather than waiting for leaf emergence. Therefore an attempt has been made to isolate the DNA from young stem. In this regard, when the DNA was isolated through M-2 method (which produced suitable DNA from leaves) poor DNA quality (0.81 to 0.96 ratio of A_{260}/A_{280}), but in good quantity (1034.38 to 1452.75 ng/µl) from the young stem was obtained (Table 1).

Table 1: Quantitative and qualitative estimation of genomic DNA isolated from the leaves and youg stem using method M-1, M-2 and M-3.

SL	Sample ID	Method M-1		Methods M-2		Methods M-3	
		Quantity of DNA (ng/µl)	Ratio of A_{260}/A_{280}	Quantity of DNA (ng/µl)	Ratio of A_{260}/A_{280}	Quantity of DNA (ng/µl)	Ratio of A_{260}/A_{280}
1	CW1 (L)	118.21	0.92	1487.12	1.87	--	--
2	CW2 (L)	112.57	0.89	1240.73	1.79	--	--
3	CW3 (L)	99.83	1.02	1402.98	1.80	--	--
	Mean ± SD	**110.20±9.42**	**0.94±0.07**	**1376.94±125.24**	**1.82±0.04**	--	--
4	CM1 (L)	132.28	0.99	1670.34	1.84	--	--
5	CM2 (L)	127.71	0.84	1485.95	1.88	--	--
6	CM3 (L)	112.49	1.01	1491.74	1.91	--	--
	Mean ± SD	**124.16± 10.36**	**0.95±0.09**	**1549 ± 104.83**	**1.88 ± 0.04**	--	--
7	CW1 (YS)	--	--	1228.74	0.87	803.31	1.81
8	CW2 (YS)	--	--	1109.37	0.92	691.74	1.86
9	CW2 (YS)	--	--	1452.75	0.81	1135.92	1.84
	Mean ± SD	--	--	**1263.62 ± 174.33**	**0.87 ± 0.06**	**876.99 ± 231.08**	**1.84 ±0.03**
10	CM1 (YS)	--	--	1356.14	0.96	1098.57	1.89
11	CM2 (YS)	--	--	1270.78	0.84	890.12	1.88
12	CM3 (YS)	--	--	1034.38	0.93	743.69	1.82
	Mean ± SD	--	--	**1220.43 ± 166.68**	**0.91±0.06**	**994.345±147.40**	**1.86±0.04**

C. wightii leave = CW1 (1) to CW3 (L); *C. wightii* Young Stem CW1 (YS) to CW3 (YS);
C. myrrah. Leaves = CM1 (L) to CM3 (L); *C. myrrah.* Young Stem = CW1 (YS) to CW3 (YS)

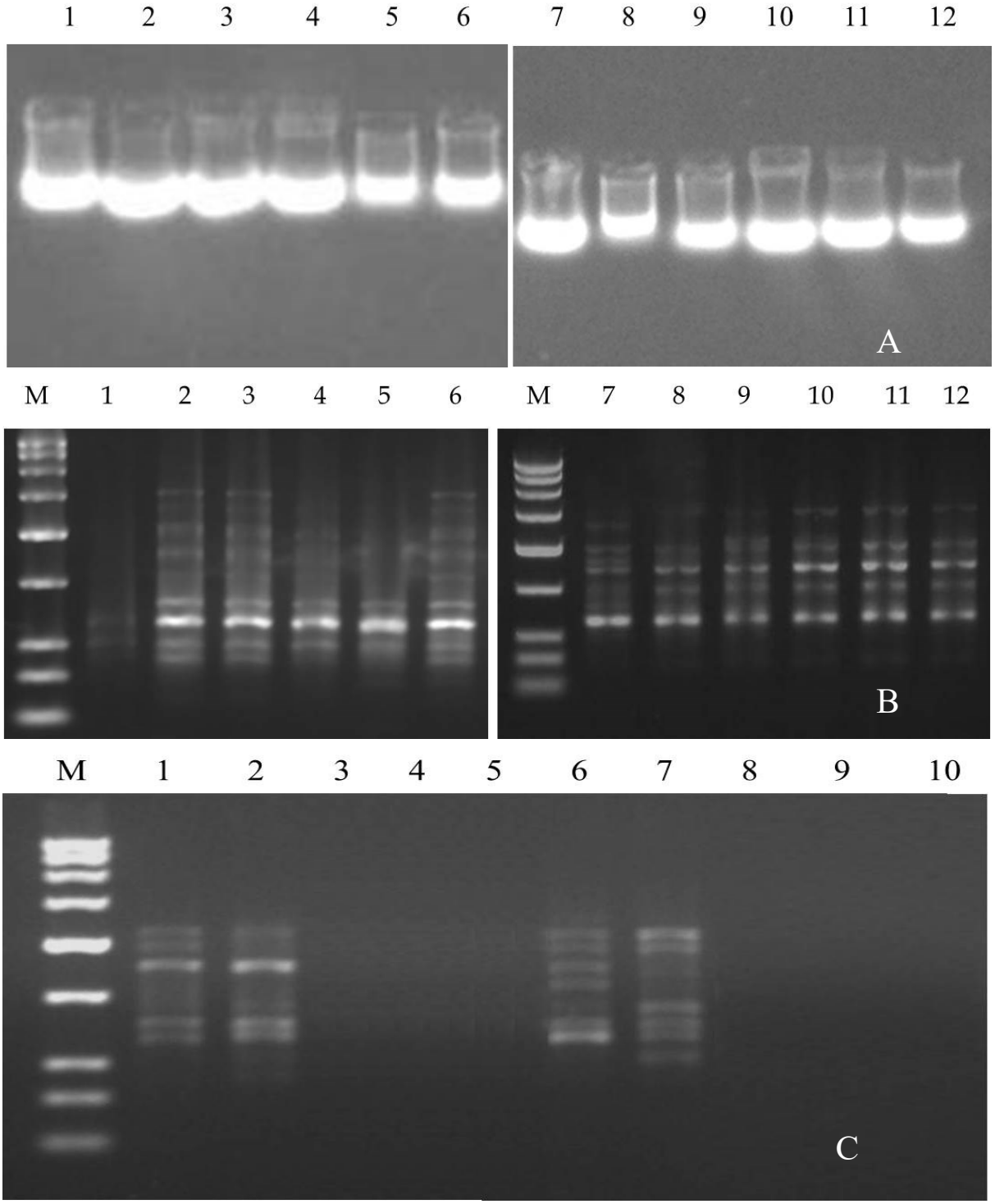

Figure 1: Isolation and Amplification of Genomic DNA of *C. wightti* and *C. myrrah.*

A. Genomic DNA isolation; Lane 1 to 3: DNA isolated from *C. wightti* leaves., **lane 4 to 6**: DNA isolated from *C. wightti* stem**, Lane 7 to 9**: DNA isolated from *C. myrrah* leaves, **lane 10 to 12**: DNA isolated from *C. myrrah* stem.

B. Isolated DNA from leaves and the stem amplified by RPI-05; Lane 1 to 3: Isolated DNA from *C. wightti* leaves, **lane 4 to 6:** Isolated DNA from *C. wightti* stem, **Lane 7 to 9:** Isolated DNA from *C. myrrah* leaves, **lane 10 to 12:** Isolated DNA from *C. myrrah* stem.

C. FTA card trapped genomic DNA amplification; Lane 1-5: amplified by RAPD primer RPI-5, **Lane 6-10:** amplified by RAPD primer RPI-10. **Lane 1 & 6**: laves was directly pressed on FTA card; **lane 2 & 7**: homogenized with PBS; **lane 3 & 8**: homogenized with DNase RNase free water; **lane 4 & 9**: finally recovered DNA of M-1 method was directly applied and **lane 5 & 10**: first precipitate of M-1 method was directly applied.

The poor quality DNA thus obtained might be due to the fact that the young stem had higher amount of polysaccharides and secondary metabolites than the leaves (Sahu *et al,* 2012) which can be overwhelmed through the treatment of higher concentration of NaCl. NaCl facilitates the removal of polysaccharides by increasing their solubility in ethanol so that they do not co-precipitate with the DNA (Fang *et al,* 1992; Choudhary *et al,* 2008). Thus to improve the DNA quality, certain modifications were created in the M-2 method. Due to this modification, the recovered DNA was good in quality (1.81 to 1.89 ratio of A_{260}/A_{280}) as well as in quantity (691.74 to 1135.92 ng/µl) (Table 1). Agarose gel electrophoresis of isolated DNA from leaves and stem using method M-2 and M-3 respectively, showed a prominent DNA band without any smearing and similarly and importantly these DNA served as best templates for good amplification with RAPD primers i.e. RPI-05 and RPI-10 (Figure 1).

The PCR amplification using DNA preserved in FTA PlantSaver card revealed that the following three sample application method i.e. direct press method where the leaves were homogenized with PBS and the leaves were homogenized with DNAse and RNAse free water, showed no amplification with RAPD primers (RPI-05 and RPI-10). This might be due to improper disruption of cell wall of plant or improper washing of the disc, because of these polysaccharides, polyphenols and other secondary metabolites retained by disc (Sairkar *et al,* 2013a). Many researchers suggested that the high concentrations of polysaccharides, polyphenols, proteins, and other secondary metabolites of plant leaves poses problem in polymerase chain reaction amplification (Koonjul *et al,* 1999; Haque *et al,* 2009; Sahu *et al,* 2012; Sairkar *et al,* 2013a).

When polysaccharide and secondary metabolites contaminated DNA precipitate from M-1 method was applied as sample on FTA PlantSaver card, a prominent PCR amplification was observed, which reveals that the major contaminants like polysaccharides, polyphenols and other secondary metabolites were washed off in M-1 method and remaining were removed during the application on FTA PlantSaver card (Figure 1).

CONCLUSION

DNA isolation of *Commiphora* species is a very tedious job for the researcher due to the presence of many secondary metabolites and unavailability of leaves all-round the year. In the present study, a suitable DNA isolation protocol was developed, in which good quality and quantity of DNA was successfully isolated from the young stem of the plant. We suggest that this DNA isolation method may also be used for other latex rich plant species. In addition to this conventional DNA isolation method, FTA PlantSaver card based DNA preservation and amplification method was developed.

Acknowledgment

The authors wish to thank Prof. P. Verma, Director General, MPCST and Dr. RK Singh, Resource Scientist and Group Head, Advance Research Instrumentation Facility, MPCST for the providing laboratory facilities.

Conflict of interest

The authors declare that there is no conflict of interest.

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International Journal of
Life Sciences
International Peer Reviewed Open Access Refereed Journal

Special Issue A 11: January 2018
UGC Approved Journal No 48951

Original Article | Open Access

Application of FTA card and novel primers for amplification and sequencing of human mitochondrial cytochrome *b* (CYTB) gene

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Manuscript details:

Available online on
http://www.ijlsci.in

ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

Editor: Dr. Arvind Chavhan

Cite this article as:
Chaudhary Ruchira, Sairkar Manisha and Sairkar Pramod (2018) Application of FTA card and novel primers for amplification and sequencing of human mitochondrial cytochrome *b* (CYTB) gene, *Int. J. of. Life Sciences*, Special Issue, A11: 31-40.

ABSTRACT

MTCYB gene produces a protein which is a component of respiratory chain complex III. This gene is also important in phylogenetic relationships between and among species. Presently many hereditary carcinomas are also associated with the alterations of this gene. The aim of the current study is to develop primers for amplification and sequencing of complete human mitochondrial cytochrome b (CYTB) gene and use of the FTA card for isolation and preservation of mitochondrial DNA. DNA from 6 blood samples were isolated by the phenol chloroform method as well as by the FTA card method which was further used for the PCR amplification and sequencing process. Novel human mitochondrial cytochrome b (CYTB) gene specific primers (two sets) were designed and examined for amplification and sequencing. DNA trapped on FTA cards and DNA isolated by the phenol chloroform method produced a brighter clear band of approximately 1320 bp with both designed primers. During sequencing reverse and forward primers of both primer sets were produced good quality DNA sequence and prepared consensus sequences covers complete cytochrome b (CYTB) gene. Whatman FTA Classic card was successfully used for storage and amplification of Human mitochondrial DNA as well as, two sets of Human mitochondrial cytochrome b (cyt b) gene specific novel primers namely MVM-MT-Cyb-01 and MVM-MT-Cyb-02.

Keywords: FTA classic card, cytochrome b gene, PCR amplification, sequencing, human mitochondria.

INTRODUCTION

Mitochondria are found in all eukaryotes, their number and location vary from single to several thousand according to cell types. Mitochondrial

(mt) genomes consist of a circular DNA molecule (16,569 base pairs) consisting of 37 genes, including 13 protein-coding genes, 2 ribosomal (r)RNA encoding genes, 22 transfer (t)RNA encodinggenes, and one major noncoding control region (Boore 1999, Chan 2006). Each mitochondrion contains 2 to 10 copies of the mtDNA. Ten times higher mutation rate was observed in *mt*DNA compared to nuclear DNA (Howell *et al,* 2003). In forensic applications, using *mt*DNA enables identification of victims, (Pereira *et al,* 2010, Pakendorf and Stoneking 2005) during mass disasters like war or terrorism (Budowlea *et al,* 2005).

*mt*DNA alterations, either deletions-insertions or base substitutions causes different cancers in human (Brandon *et al,* 2006), including breast cancer (Plak *et al,* 2009), ovarian cancer (Aikhionbare *et al,* 2007), colorectal cancer (Aikhionbare *et al,* 2004), gastric cancer (Bi *et al,* 2011, Sui *et al,* 2006), hepatic cancer (Yin *et al,* 2010), lungs cancer (Hosgood *et al,* 2010) prostate cancer (Ray *et al,* 2009), thyroid cancer (Gasparre *et al,* 2010) and head and neck cancer (Allegra *et al,* 2006).

MTCYB is encoded by the guanine-rich heavy (H) strand of the *mt*DNA and located between nucleotide pairs (nps) 14747 and 15887 (Anderson *et al,* 1981; Wallace 1994). The *MTCYB* gene encompasses 1140 nps of *mt*DNA and encodes a single polypeptide without introns. In the mitochondrion of eukaryotes, cytochrome *b* produces a protein which is a component of respiratory chain complex III (EC 1.10.2.2) also known as Ubiquinol-cytochrome c reductase. These are involved in electron transport, pumping of protons to create a Proton Motive Force (PMF). The proton gradient is finally used for the generation of ATP. Cytochrome *b* is an integral membrane protein of approximately 400 amino acid residues that has 8 transmembrane segments. Four conserved histidine residues are postulated to be the ligands of the iron atoms of these two heme groups (Howell 1989; Esposti *et al,* 1993).

Comparison and identification of species in the same genus or the same family could be well studied by an aide of sequence variability of cytochrome *b* (Tsai *et al,* 2007). Phylogenetic studies of cytochrome *b* gene propose new classification schemes that better reflect the phylogenetic relationships among the species (Schall and Denis 2010, Castresana 2001). Alteration in the *MTCYB* gene causes many diseases like hypertrophic cardiomyopathy (Valnot *et al,* 1999; Andreu 2000; Hagen *et al,* 2013) mitochondrial myopathies (Andreu *et al,* 1999) various tumors (Fliss *et al,* 2000; Liu *et al,* 2001), bladder cancer (Dasgupta *et al,* 2008) heteroplasmic (Legros *et al,* 2001) chronic lactic acidosis (Mancuso *et al,* 2003) etc.

FTA technology is a novel method designed to simplify the collection, shipment, archiving and purification of nucleic acid from a wide variety of biological sources (Yoshida *et al,* 1995). The FTA™ purification method is very mild and similar to detergent based protocols (Montgomery and Sise, 1990) compared to traditional phenol based purification methods (Maniatis et al, 1989, Mullen *et al,* 2009).

In this study, genomic DNA isolated through FTA card and phenol chloroform method was used for the amplification of human mitochondrial cytochrome *b* gene. Two primer sets were designed for amplification and sequencing of complete human mitochondrial cytochrome *b* (cyt *b*) gene.

MATERIALS AND METHODS

Collection and storage of samples

A total of 6 samples were collected from Cancer Hospital and Research Centre Gwalior, Madhya Pradesh, India in the form of fresh blood. Fresh blood was collected in EDTA tubes and stored at 4°C. FTA classic card (Whatman Inc., 2009) was used for the storage of samples at room temperature. About 100 µl of blood sample was applied to labelled FTA card and allowed to dry at room temperature. These cards were directly used for PCR amplification of genomic DNA.

Isolation of genomic DNA from Whole Blood using the phenol chloroform method

About 0.5 mL of blood sample was taken and 1.5 mL of erythrocyte lysis buffer I (10 mM Tris HCl (pH 8.0), 320 mM Sucrose, 5 mM $MgCl_2$, 1% Triton X) was added. This mixture was centrifuged at 3600 rpm for 10 minutes and the supernatant was discarded. Precipitate was dissolved in 0.8 mL of lysis buffer II (400 mM Tris HCl, 60 mM EDTA, 150 mM NaCl, 1%SDS added after autoclaving) and 0.2 mL of 5M sodium perchlorate by vigorous shaking. When precipitate dissolved completely, an equal volume of Phenol:Chloroform: Isomylalcohol (25:24:1) was added, mixed gently and centrifuged at 3000 rpm for 10 minutes. The aqueous

layer so formed was collected in a fresh centrifuge tube, mixed thoroughly with equal volume of chloroform and centrifuged at 3000rpm at 10 minutes. The aqueous layer was collected again in a fresh centrifuge tube and ice cold ethanol was added to precipitate DNA. The precipitated DNA was washed twice with 70% ethanol, air dried and dissolved in 0.1 mL TE buffer (10 mM Tris HCl (pH 7.5), 1m M EDTA, pH 8.0). Quantity and quality of isolated DNA were measured using spectrophotometer (Sambrook and Russell, 2001).

Isolation of genomic DNA from Whole Blood using FTA Card

Three discs were removed from the center of the FTA card using the 2.0 mm Harris Micro Punch tool and collected in 200 µl PCR amplification tubes. About 200 µl FTA purification reagent was added to each tube, capped and inverted twice and incubated for 5 minutes at room temperature. FTA reagent was pipetted up and down twice and discarded as much reagent as possible and this step was repeated once again with 200 µl of FTA reagent. Then 200 µl of TE Buffer was added and inverted twice and incubated at room temperature for 5 minutes. Finally discs were allowed to completely air dry for 1 hour at room temperature (Whatman Inc., 2009).

Design of human mitochondrial cytochrome *b* (CYTB) gene specific primers

The reference sequence of human mitochondrial DNA (ref: NC_012920.1, GI: 251831106) was downloaded from NCBI database and a fragment of this reference sequence (location of mitochondrial DNA: 14500 to 16200) was used for designing of cytochrome *b* gene specific primers. The parameters like, 1200-1400 bp product length, 19 to 26 bp (optimum 20 bp) primer size, up to 60% GC contents, melting temperature of the primer (Tm) between 57 to 61^0C were followed during designing of primers by PRIMER 3 software (Rozen and Skaletsky 2000).

Amplification and sequencing of *MTCYB* gene

The designed *MTCYB* gene specific primers were examined by amplification of cytochrome *b* gene through polymerase chain reaction. 50 µl of the PCR reaction consisting of 25 µl 2x red dye PCR mix, 1 µl (10 pico mole) each reverse and forward primers and 22 µl sterile DNAse, RNAse free water with 1 µl (25ng/ 1µl) of the isolated genomic DNA. In the case of FTA Card method, instead of genomic DNA, 3 purified FTA discs were added.

Amplification was performed on the automatic thermal cycler (ABI) and the PCR conditions consisting of an initial denaturation (one cycle) at 94 °C for 6 minutes, 30 cycles of 1 minute at 94°C, 1 minute at 59°C and 2 minutes at 72°C and a final extension at 72°C for 10 minutes (Matsuda *et al,* 2005 Anderson *et al,* 1981). About 5 µl of PCR products were resolved on 1 % agarose gel with 100bp DNA ladder and remaining PCR product were purified using Medox-Easy Spin Column PCR Cleanup Minipreps kit. These purified PCR products were sequenced at Samved BioTech Pvt. Ltd. Ahmedabad.

Data analysis

Obtained DNA sequence was visualized and analyzed by software SeqScanner 2 including quality of the sequence, Contagious read length (CRL) Q+16 values. Consensus sequences were prepared by MEGA6 (Tamura et al, 2013) software. Similar sequences were searched and annotated using online software BLAST (Basic Local Alignment Search Tool) provided by NCBI (National Centre of Biotechnology Information). Examined primers were submitted in probe database and DNA sequences were submitted in Gene bank database of NCBI.

RESULTS

DNA Isolation from fresh blood by traditional method

Genomic DNA was isolated in triplicate from each of the six samples by the traditional phenol chloroform method. The quantity of obtaining genomic DNA was in the range of 128.27±4.0197 to 185.58±10.9378, while ratio of A_{260}/A_{280} was observed between 1.73±0.0153 to 1.89 ±0.0208 (Table 1). These DNA produces prominent and clear band on 1.5% agarose gel.

Designing of Novel Primers

Reference sequence of *Homo sapiens* mitochondrion genomic DNA (ref: NC_012920.1, GI: 251831106) was downloaded from the NCBI gene bank in the FASTA format. This sequence was trimmed from both 5' and 3' ends and finally a 1400 bp sequence (ref location: 14501 to 15900) covering complete Cytochrome *b* gene was obtained. Using this sequence, specific primer was designed for amplification of complete *MTCYB* gene by Primer3 software. Two suitable sets of reverse and forward primers were selected from the list of primers and named as MVM-MT-Cyb-01 and MVM-MT-Cyb-02.

These primers were 19 to 21 bases long and their melting temperature was in the range of 64.9 to 65.5 ˚C. The product sizes of MVM-MT-Cyb-01 and MVM-MT-Cyb-02 primers were 1312 and 1317 bp respectively (Table 2).

Examination of designed primers

MVM-MT-Cyb-01 and MVM-MT-Cyb-02 were synthesized and supplied by Sigma Aldrich, India. DNA samples isolated by the phenol chloroform method and trapped on FTA cards were amplified for the authentication of developing novel primers. Amplified PCR products were resolved on 1.5% agarose gel and the result revealed that the both the primers produced prominent band of approximately 1320 bp in all the screened samples.

Sequencing of *MTCYB* gene using developed primers

The amplified product of six samples was sequenced using reverse and forward primers of both set MVM-MT-Cyb-01 and MVM-MT-Cyb-02. Contiguous read length (CRL) of sequences obtained by reverse primer of MVM-MT-Cyb-01 was observed in the range of 898 to 1048 bp with a mean of 957.667± 61.976 bp and in the range 987 to 1043 bp with a mean of 1022.833± 22.825 by forwarded primer of MVM-MT-Cyb-01. While CRL of sequences obtained by reverse primer of MVM-MT-Cyb-

Table 1: Quantitative and qualitative estimation of isolated DNA

SL	Sample ID	Ratio of A_{260}/A_{280}	Concentration (ng/µl)
1.	B-01	1.76±0.0116	128.27 ±4.0197
2.	B-02	1.81±0.0306	185.58 ±10.9378
3.	B-03	1.89±0.0208	163.76 ±2.8725
4.	B-07	1.73±0.0153	175.15 ±2.1854
5.	B-08	1.75±0.0321	169.15 ±2.4856
6.	B-09	1.79±0.0265	182.15 ±3.1075

Table 2: Sequence of designed primers

SL	Name	Code	Sequence (5'--------3')	Total Length	Temp.	Product size
1.	MVM-MT-Cyb-01	P1-f	CACGGACTACAACCACGACC	20 bases	65.5 ˚C	1312 bp
		P1-r	TTGGGTGCTAATGGTGGAGTT	21 bases	65.4˚C	
2.	MVM-MT-Cyb-02	P2-f	TCGCACGGACTACAACCAC	19 bases	64.9˚C	1317 bp
		P2-r	CTTTGGGTGCTAATGGTGGAG	21 bases	65.4˚C	

Table 3: Consensus sequences of primer MVM-MT-Cyb-01 and MVM-MT-Cyb-02

SL	Sample ID	Obtained Sequences (bp) by Primer MVM-MT-Cyb-01			Obtained Sequences (bp) by Primer MVM-MT-Cyb-02		
		Reverse (CRL)	Forward (CRL)	Consensus sequences	Reverse (CRL)	Forward (CRL)	Consensus sequences
1.	B-01	898	1027	1140	941	1123	1259
2.	B-02	1010	1041	1180	1072	1056	1248
3.	B-03	945	1036	970	1043	1035	1011
4.	B-07	1048	1003	1082	1073	1130	1251
5.	B-08	890	987	1011	991	1236	1246
6.	B-09	955	1043	1170	949	1134	1262
Mean±SD		957.667± 61.976	1022.833± 22.825	1092.167± 86.806	1011.5± 59.551	1119± 70.818	1203± 99.075

02 were recorded in the range of 941 to 1073 bp with a mean of 1011.5± 59.551 bp and forwarded primer produced 1035 to 1236 bp with a mean of 1119± 70.818 bp.

The contiguous read length (CRL) is the longest uninterrupted stretch of bases with quality, higher than a specified limit (QV16+). In the evaluation of the quality of each base, not only the best quality value of that base was used, but also those of adjacent bases within a specified window size. According to the contiguous read length (CRL) both the developed primers were produced good quality of readable sequences. 1092.167± 86.806 bp mean consensus sequence with minimum 970 and maximum 1180 bp and 1203± 99.075 bp mean consensus sequence with minimum 1011 and maximum 1262 bp were prepared using reverse and forward primers of primer MVM-MT-Cyb-01 and primer MVM-MT-Cyb-02 respectively (Table 3).

Annotation and submission

Similar sequences of obtained sequences were searched in the gene bank database (NCBI) using BLAST software one by one. Obtained sequences were annotated as per provided information by BLAST search. According to that 3 tRNA region, namely TRNE (tRNA- Glutamine; GeneID:4556; HGNC:7479; MIM:590025), TRNT (tRNA-Threonine; GeneID:4576; HGNC:7499; MIM:590090) and TRNP (tRNA-Proline; GeneID:4571; HGNC:7494; MIM:590075) and one coding region (gene- MTCYB; GeneID:4519; HGNC:7427; MIM:516020) were observed in obtained sequences. Out of 6 obtained sequences all sequences having TRNE gene, which was partially at 3' end. According to BLAST search gene- *MTCYB* was observed in obtained sequences which produces protein Cytochrome *b* (protein_id="YP_003024038.1"). Gene *MTCYB* was observed in the all obtained sequences, out of them complete gene or coding region was observed in 5 sequences. A TRNT gene was observed in 5 obtained sequences. TRNP gene was observed in 5 obtained sequences. After successful annotation of the sequences, these sequences were submitted in NCBI gene bank database and gene bank ID was allotted for all 6 sequences. Furthermore the information of the sample used in this study was also submitted in the NCBI BioSample database.

Wet laboratory authenticated primers were submitted in the NCBI probe database after successful validation of wet laboratory amplification and sequencing. After submission, accession no Pr032250587 with Probe ID 32250587 was assigned to primer set MVM-MT-Cyb-01 and accession no Pr032250588 with Probe ID 32250588 was assigned to primer set MVM-MT-Cyb-02.

DISCUSSION

Blood is the main source of DNA for genotype-related studies in humans. A rapid, efficient, and cost-effective method for the isolation of genomic DNA from whole blood is needed for screening a large number of samples (Subbarayan *et al,* 2002). Phenol chloroform method for genomic DNA isolation showed good quality and quantity. These methods are reliable and have a long history of isolation of genomic DNA from human blood samples (Parzer and Mannhalter 1991; Albarino and Romanowski 1994; Wang 1994; Robbins *et al,* 1995; Rudbeck and Dissing 1998; Sambrook and Russell 2001). Time to time, many researchers made various alterations in this method for recovering DNA from different part of the human body, like white blood cells (Ikuta *et al,* 1992), buccal cells (Cozier *et al,* 2004) buccal swabs (Walker *et al,* 1999), formalin–fixed paraffin–embedded tissue sections (Rivero *et al,* 2006).

In this study FTA classic card was used for preservation of genomic DNA at room temperature. These FTA cards were successfully used for amplification of mitochondrial DNA. According to the best of our knowledge, this is the first report in which mitochondrial DNA was amplified using the FTA card. FTA is a simple technology that reduces the steps of DNA collection, transportation, purification and storage and subsequently reduces the cost and time required to process DNA to the final step of purified DNA ready for the downstream application (Rajendram *et al,* 2006; Whatman Inc., 2009). The FTA cards are firstly developed for Phenylketonuria screening in newborns by Guthrie and Susi in 1963, later these cards are used in medical and forensic science for detecting DNA using the polymerase chain reaction (Vanek *et al,* 2001). It has been also used for isolation of genomic DNA from animal samples (Crabbe 2003; Harvey 2005; Borisenko *et al,* 2008), bacterial DNA or viral RNA from different biological samples (Lampel and Orlandi 2002; Lampel *et al,* 2004; Warren *et al,* 2005). Presently FTA cards are used in both DNA and RNA based studies of many plants (Roy and Nassuth 2005; Tsukaya *et al,* 2005; Adugna *et al,* 2011; Sairkar *et al,* 2013).

In this study, the mitochondrial cytochrome *b* gene was amplified for detection of SNPs in breast and ovarian

carcinoma. Cytochrome *b* of eukaryotic mitochondria (*MTCYB*) is an important enzyme of respiratory chain complex III, also known as the bc1 complex or Ubiquinol-cytochrome c reductase. *MTCYB* is an imperative membrane protein of 380 aminoacid that possibly has 8 transmembrane segments (Howell 1989; Esposti *et al,* 1993). The DNA sequence of *MTCYB* is commonly used to determine phylogenetic relationships between organisms due to its sequence variability. It is considered to be most useful in determining relationships within families and genera (Castresana 2001). *MTCYB* has been used for a diversity assessment at the species level (Meyer and Wilson, 1990; Irwin *et al,* 1991; Normark *et al,* 1991; Cantatore *et al,* 1994; Lydeard and Roe 1997; Kumazawa and Nishida 2000) and at the population levels (Sturmbauer and Meyer 1992; Rocha-Olivares *et al,* 1999; Kirchman *et al,* 2000; Lovejoy and de Arau´jo 2000). The phylogenetic effectiveness of the *MTCYB* gene has been studied at several taxonomic levels among animals taxa (Irwin *et al,* 1991; Moritz *et al,* 1992; da Silva and Paton 1993; Graybeal 1993; Lamb and Lydeard 1994; Moore and DeFilippis 1997; Nunn and Stanley 1998) and particularly in fish taxa (Ortí and Meyer 1996, 1997; Lydeard and Roe 1997; Martin and Bermingham 1998; Zardoya and Doadrio 1999; Lovejoy and de Arau´jo 2000).

In this study two primers were developed for amplification of complete *MTCYB* gene, which produced 1312 and 1317 bp fragments and these primers were also successfully used for capillary based Sanger sequencing. In this study, 3 tRNA regions, namely TRNE (tRNA- Glutamine), TRNT (tRNA- Threonine) and TRNP (tRNA-Proline) and one coding region CDS (gene- *MTCYB*) were observed in obtained sequences. These results revealed that the developed primers may be used for amplification and sequencing of complete CDS regions. In previous studies, many primers were developed and used for amplification of a *MTCYB* like, Kocher *et al,* (1989) developed a set of primers which amplified a 307-base-pair segment of the cytochrome *b* gene not only from humans but also from most other vertebrates. Kent and Norris (2005) developed a specific multiplexed primer set based on mitochondrial cytochrome *b*. A "universal" primer pair has been designed and validated to amplify a 464 bp segment of the cytochrome *b* gene (Unseld *et al,* 1995) while Edwards *et al,* (1991), Krajewski *et al,* (1992) and Krajewski and Fetzner (1994) developed two primer sets each for amplification of mitochondrial cytochrome *b* gene.

Universal PCR primers generated several different anomalous sequences during amplifying a portion of the cytochrome *b* gene and this force the researcher to believe it to be nuclear pseudogenes (Mirol *et al,* 2000; Smith *et al,* 1992; Arctander, 1995; Collura *et al,* 1996; Zhang & Hewitt, 1996a, b). This problem may be overcome by complete amplification of gene and in this study, we amplified 1317 bp fragment which contained tRNA region, namely TRNE (tRNA- Glutamine; GeneID:4556; HGNC:7479; MIM:590025), TRNT (tRNA-Threonine; GeneID:4576; HGNC:7499; MIM:590090), TRNP (tRNA-Proline; GeneID:4571; HGNC:7494; MIM:590075) and one coding region (gene- *MTCYB*; GeneID:4519; HGNC:7427; MIM:516020). Amplification of *MTCYB* gene using our developed primers may reduce the chances of confusion between nuclear and mitochondrial copies.

CONCLUSION

In the present study, two novel primers were developed for amplification and sequencing of *MTCYB* gene. We conclude that, the FTA card is suitable for amplification of mitochondrial DNA as well as suitable for the preservation of DNA at room temperature. These primers and FTA card may also be used for study of large samples with complete mitochondrial DNA.

Acknowledgment

The authors are grateful to Dr. (Mrs) Abha Gargav, Principal, Govt. Motilal Vigyan Mahavidyalaya, for providing the opportunity to work in this prestigious academic institution. The authors also like to thank to Dr. BR Shrivastav, Director, Cancer Hospital & Research Institute (CHRI), Gwalior and Dr. Archana Shrivastav, Principal, College of Life Sciences, Gwalior for providing blood samples.

Conflicts of interest: The authors stated that no conflicts of interest.

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International Journal of
Life Sciences
International Peer Reviewed Open Access Refereed Journal

Special Issue A 11: January 2018
UGC Approved Journal No 48951

Original Article Open Access

Molecular assessment of Coronary Heart Disease(CHD) risk in obese and overweight subjects

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Manuscript details:

Available online on
http://www.ijlsci.in

ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

Editor: Dr. Arvind Chavhan

Cite this article as:
Jomy TJ and Lakshman Kumar B (2018) Molecular assessment of Coronary Heart Disease(CHD) risk in obese and overweight subjects, *Int. J. of. Life Sciences*, Special Issue, A11: 41-48.

ABSTRACT

Coronary artery disease is the most common type of heart disease for which obesity is known to be one of the causative factors. The present study was thus carried out to determine the distribution of SNP (single nucleotide polymorphism) rs3737787 in upstream stimulatory factor 1 (USF1) gene and SNP rs1130864 of C-reactive protein (CRP) gene in 25 obese and overweight blood samples (based on BMI) collected from Erode district, Tamil Nadu along with equal number of healthy samples. The methodology used for the study was PCR-SSCP followed by DNA sequencing. In the present study for the polymorphisms studied, both the controls and patients showed more or less similar distribution of genotypes and hence they could not be ascertained for coronary heart disease risk in the subjects studied. But for further confirmation, a higher sample size needs to be studied.

Keywords: Coronary artery disease, C-reactive protein, Upstream stimulating factor 1, SNP rs1130864, SNP rs3737787, BMI

INTRODUCTION

Obesity is a term used to describe body weight that is much greater than what is healthy. Adults with a body mass index (BMI, calculated as weight in kilograms divided by height in meters squared) between 25 kg/m^2 and 30 kg/m^2 are considered overweight. Adults with a BMI greater than or equal to 30 kg/m^2 are considered obese. Anyone who is more than 100 pounds overweight or who has a BMI greater than or equal to 40 kg/m^2 is considered morbidly obese.

Genetic factors play some part in the development of obesity. Children of obese parents are 10 times more likely to be obese than children with parents of normal weight (Leslie *et al*., 2007). The upstream transcription factor 1 (USF1) gene encoding USF1, a ubiquitously expressed transcription factor controlling some 40 genes (Naukkarinen *et al*., 2005).

The product of *USF1* regulates numerous genes of lipid and glucose metabolism (Choquette *et al*., 2007), and in large population cohorts specific alleles of *USF1* are associated with the risk of cardio vascular disease (CVD) and based on its function is an attractive candidate gene for CVD (Komulainen *et al*., 2006).

C-reactive protein (CRP) levels are associated with CHD in healthy subjects, both in a cross-sectional study in general practice (Mendall *et al*., 1996), and longitudinal-lly in the US Physicians Health Study (Ridker *et al*., 1997), the MONICA-Augsburg Cohort Study (Koenig *et al*., 1997), and the MRFIT Study (Kuller *et al*., 1996), where CRP levels predicted cardiovascular events or CHD mortality during a follow-up of between 2 and 17 years. The T-allele of SNP1444C>T (rs 1130864) has been reported to affect both baseline CRP and inflame-matory responses to experimental lipopolysaccharide-induced endotoxemia in healthy adults (Marsik *et al*., 2006). This allele is also associated with differential CRP responses in patients undergoing periodontal treatment and coronary artery bypass graft surgery (Brull *et al*., 2003). The present study was conducted to analyze previously reported SNPs of USF-1 and CRP in the population studied and check their association with the manifestation of the condition.

MATERIALS AND METHODS

Study participants

Twenty five blood samples were collected from Obese and overweight subjects and equal number of healthy normal controls from 'Deepa Micro Lab', Erode, Tamil Nadu. The ethical clearance for the work was obtained from Ethical committee, PSGIMSR (PSG Institute for Medical Sciences and Research) Coimbatore, Tamilnadu.

Genomic DNA extraction

One mL of whole blood was lysed with 3mL of chilled RBC lysis buffer, vortexed for 1 minute and centrifuged at 4000rpm for 5 minutes and the red supernatant was removed. This step was repeated twice to get white to pink pellet. To this 200µL of nuclei lysis buffer and 50µL of SDS were added. Followed by the addition of 3µL of proteinase K, the mixture was incubated at 65^{o}C for 2:30 hours. After this, 175µL of 5.3M Sodium chloride was added, centrifuged at 10,000rpm for 15 minutes, supernatant carefully siphoned off and transferred to a new 2 mL microcentrifuge tube. To this one mL of cold 100% ethanol was added and inverted ten times to precipitate the DNA. Thereafter the tube was centrifuged at 1500 rpm for 10 minutes, supernatant was removed and the pellet was resuspended in 75% alcohol. Centrifugation at 15000 rpm was performed to remove supernatant. The pellet was air dried and resuspended in 100-150µL of TE buffer and stored at -20^{0}C. The A_{260}/A_{280} values were checked to assess DNA purity through UV/Visible spectrophotometer.

PCR amplification and analyses of USF-1 (rs3737787) and CRP (rs1130864) +1444C>T SNPs

USF-1 (rs3737787) SNP was determined using the following primer pairs, forward and reverse primer sequences were as follows, forward 5'-GGCCTGCAGTGG TGTGAAA-3' and reverse 5-'TCCAGTATCCAGCATGGA GA CA-3'. CRP (rs1130864) +1444C>T polymorphism was assessed using previously reported primers and the sequence as follows, forward 5'-GTGTCTGGTCTGGGAGC TCGTTA-3' and reverse 5'CTTCTCAGCTCTTGCCTTATGA GT-3'. Thermal condi-tions for USF-1 consisted of Initial denaturation at 94^{o}C for 10 mins, 30 cycles of denature-tion at 94^{o}C for 1 min, Annealing at 62.1^{o}C for 45 Sec, extension at 72 for 1 min and final extension at 72^{o}C for 10 mins. Thermal conditions for amplification of CRP gene differed only in annealing which was 54^{o}C and the rest similar to thermal conditions of USF-1 gene.

Single Strand Conformational (SSCP) analysis of USF-1 and CRP

About 7µL of PCR amplicons (USF-1 / CRP) were taken and mixed with 15µL of loading dye. This mixture was denatured at 95^{o}C for 6 mins and immediately kept on ice to avoid renaturation and loaded on 10% PAGE. Silver staining (0.2% silver nitrate) method was used to stain DNA and viewed. The gel showing abnormal band pattern was confirmed by sequencing.

DNA sequencing

The abnormal bands observed in SSCP analysis were sequenced using an Automated DNA sequencer (ABI Prism, Chromous Biotech Pvt. Ltd, Bengaluru).

RESULTS

Based on the BMI, there were 18 obese and seven overweight subjects (Table 1).

PCR-SSCP analysis of USF-1 and CRP genes

PCR amplification of USF-1 gene resulted in 129 bp amplicon (Fig. 1). Of the 50 samples analyzed for USF-1

Table 1: Subjects recruited and categorized as obese, overweight or normal based on BMI

Samples	Weight in kg	Height in metre squared	BMI (in kg per metre square)	Category
S1	88	2.62	33.58	Obese
S2	70	1.96	35.71	Obese
S3	80	1.96	40.81	Obese
S4	60	2.4	25	Overweight
S5	75	2.25	3.33	Obese
S6	65	1.19	33.16	Obese
S7	55	2.01	27.36	Overweight
S8	45	2.4	18.75	Normal
S9	50	1.96	25.51	Overweight
S10	40	1.96	20.4	Normal
S11	43	2.04	21.07	Normal
S12	52	1.93	26.96	Overweight
S13	40	1.82	21.96	Normal
S14	45	1.74	29.88	Overweight
S15	84	2.62	32.06	Obese
S16	70	1.96	36.22	Obese
S17	90	2.62	34.35	Obese
S18	62	2.62	23.66	Normal
S19	63	2.52	25	Overweight
S20	59	2.59	22.77	Normal
S21	60	2.52	23.80	Normal
S22	71	1.96	36.22	Obese
S23	84	2.01	41.79	Obese
S24	65	2.04	31.86	Obese
S25	45	2.04	22.05	Normal
S26	48	2.25	21.33	Normal
S27	50	2.4	20.83	Normal
S28	61	1.96	31.12	Obese
S29	71	2.62	27.09	Overweight
S30	70	2.04	34.31	Obese
S31	48	2.04	23.52	Normal
S32	52	2.62	19.84	Normal
S33	58	2.52	23.01	Normal
S34	62	2.04	30.39	Obese
S35	63	2.62	24.04	Normal
S36	72	1.96	36.73	Obese
S37	93	2.52	36.90	Obese
S38	90	2.62	34.35	Obese
S39	80	2.04	39.21	Obese
S40	46	2.4	19.16	Normal
S41	42	1.96	21.42	Normal
S42	41	1.96	20.91	Normal
S43	45	2.04	22.05	Normal
S44	61	2.52	24.2	Normal
S45	51	2.4	21.25	Normal
S46	43	1.82	20.99	Normal
S47	52	1.74	23.5	Normal
S48	63	1.66	22.0	Normal
S49	60	1.80	21.0	Normal
S50	75	1.92	20.5	Normal

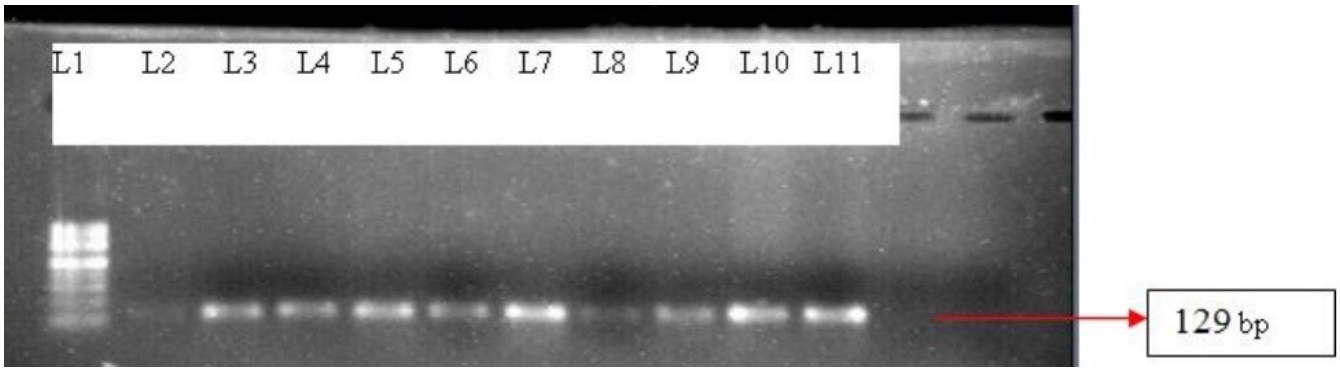

Figure 1: USF1 gene fragment amplified in obese and overweight samples

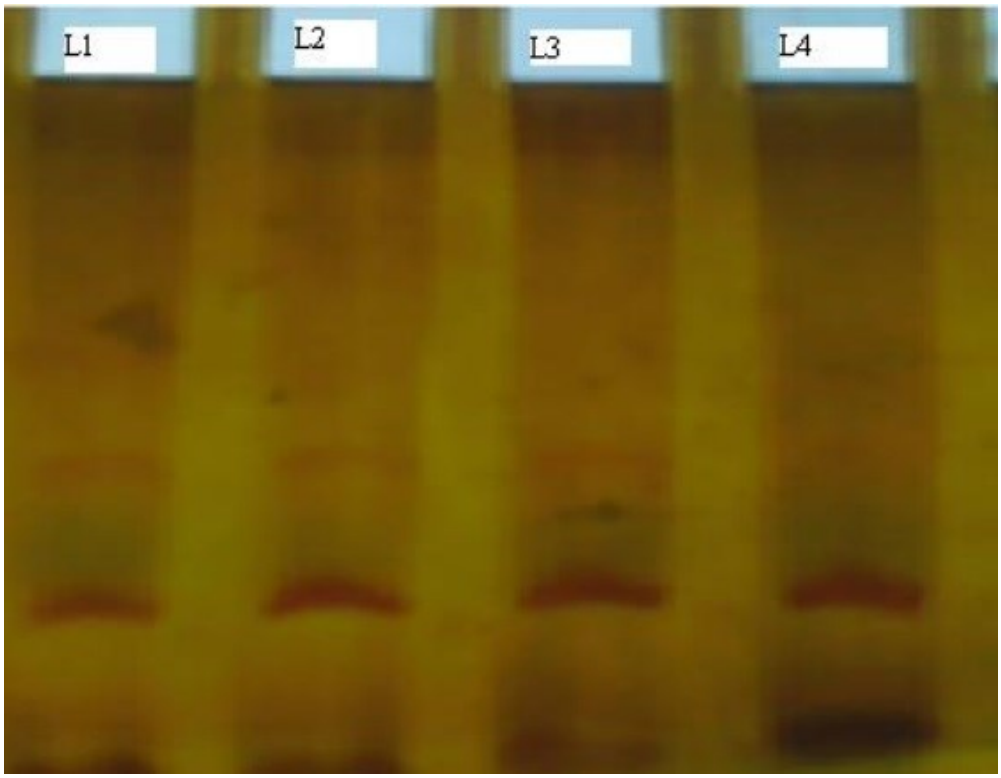

Figure 2: USF-1 gene PCR-SSCP abnormal band pattern in lane 4 (An obese patient)

Sequence ID: lcl|Query_37757 Length: 170 Number of Matches: 1

Range 1: 35 to 170 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
252 bits(136)	2e-72	136/136(100%)	0/136(0%)	Plus/Plus

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Sbjct  35   GGAGACAGGCCTGCAGTGGTGTGAAACACACAATGTGGACGTGCACTGACAGCCTTGCCC  94

Query  61   ACCCCCACCATGCAGCCCCTGGGCCCTTGTGCTCCTCTCGCACAATGCATGTGCTGTCTC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  95   ACCCCCACCATGCAGCCCCTGGGCCCTTGTGCTCCTCTCGCACAATGCATGTGCTGTCTC  154

Query  121  CATGCTGGATACTGGA  136
            ||||||||||||||||
Sbjct  155  CATGCTGGATACTGGA  170
```

Figure 3: Blast alignment of USF1 sequence in an obese subject showing the CC homozygote

Sequence ID: lcl|Query_39365 Length: 170 Number of Matches: 1

Range 1: 35 to 170 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
246 bits(133)	1e-70	135/136(99%)	0/136(0%)	Plus/Plus

```
Query  1    GGAGACAGGCCTGCAGTGGTGTGAAACACACAATGTGGATGTGCACTGACAGCCTTGCCC  60
            ||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  35   GGAGACAGGCCTGCAGTGGTGTGAAACACACAATGTGGACGTGCACTGACAGCCTTGCCC  94

Query  61   ACCCCCACCATGCAGCCCCTGGGCCCTTGTGCTCCTCTCGCACAATGCATGTGCTGTCTC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  95   ACCCCCACCATGCAGCCCCTGGGCCCTTGTGCTCCTCTCGCACAATGCATGTGCTGTCTC  154

Query  121  CATGCTGGATACTGGA  136
            ||||||||||||||||
Sbjct  155  CATGCTGGATACTGGA  170
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Figure 4: Blast alignment of USF1 sequence in an obese subject showing the CT heterozygote

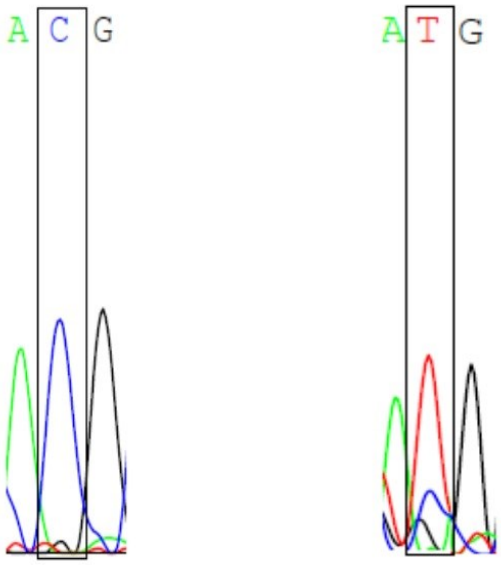

Figure 5: Homozygous CC and heterozygous CT change in USF1 gene as visualized on chromatogram present in obese subjects

SNPs through SSCP, three samples were found to posses abnormal bands and all of these were either obese or overweight (Fig.2). Blast analysis of the sequence is as given in figure 3 and figure 4. The predominant genotype observed among patients and controls was CC homozygous type. The heterozygous CT was the second genotype present in patients and controls as interpreted in the chromatogram (Fig. 5).

PCR amplification with CRP specific primers yielded a 195 bp amplicon (Fig. 6) and of the 50 samples analyzed by PCR-SSCP, two patients found to posses abnormal bands (Fig 7). Blast analysis is as shown in figure 8 and figure 9. Predominant genotype in SNP of CRP gene was found to be CC followed by CT as interpreted in the chromatogram (Fig.10). With respect to both USF-1 and CRP genes, the distribution of allele was more or less similar between obese or overweight patients and controls (Table 2). Hence, the current study could not associate any genotypes with the disease risk in patients.

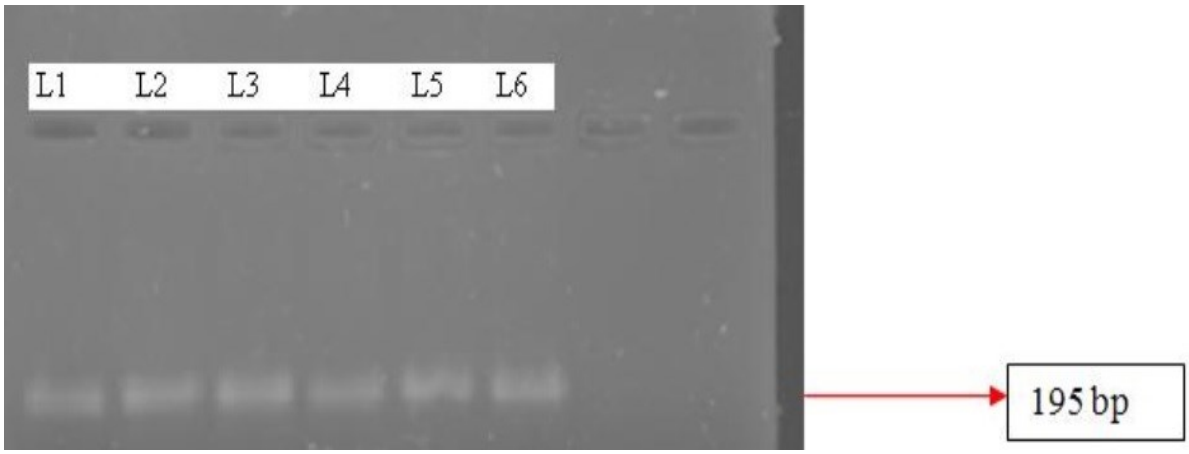

Figure 6: CRP gene fragment amplified in obese and overweight subjects

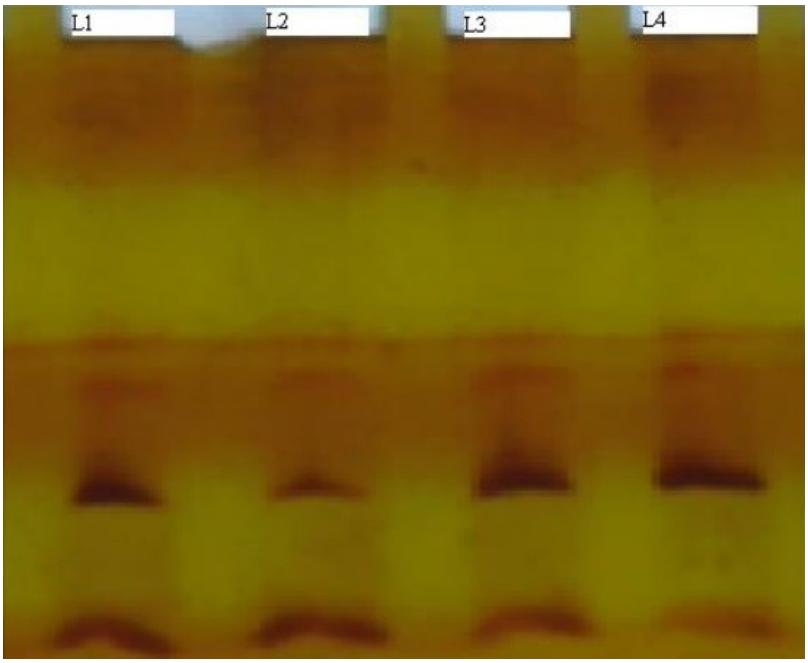

Figure 7: CRP gene SSCP abnormal band pattern in lane 2 (an obese patient)

Sequence ID: lcl|Query_57287 Length: 244 Number of Matches: 1

Range 1: 70 to 243 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
322 bits(174)	3e-93	174/174(100%)	0/174(0%)	Plus/Plus

```
Query  1    TAACTATGCTGGGAAACGGTCCAAAAGAATCAGAATTTGAGGTGTTTTGTTTTCATTTTT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  70   TAACTATGCTGGGAAACGGTCCAAAAGAATCAGAATTTGAGGTGTTTTGTTTTCATTTTT  129

Query  61   ATTTCAAGTTGGACAGATCTTGGAGATAATTTCTTACCTCACATAGATGAGAAAACTAAC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  130  ATTTCAAGTTGGACAGATCTTGGAGATAATTTCTTACCTCACATAGATGAGAAAACTAAC  189

Query  121  ACCCAGAAAGGAGAAATGATGTTATAAAAAACTCATAAGGCAAGAGCTGAGAAG  174
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  190  ACCCAGAAAGGAGAAATGATGTTATAAAAAACTCATAAGGCAAGAGCTGAGAAG  243
```

Figure 8: Blast alignment of CRP sequence in a patient showing the CC homozygote

Download Graphics

Sequence ID: lcl|Query_180591 Length: 244 Number of Matches: 1

Range 1: 70 to 243 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
316 bits(171)	2e-91	173/174(99%)	0/174(0%)	Plus/Plus

```
Query  1    TAACTATGCTGGGAAATGGTCCAAAAGAATCAGAATTTGAGGTGTTTTGTTTTCATTTTT  60
            ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  70   TAACTATGCTGGGAAACGGTCCAAAAGAATCAGAATTTGAGGTGTTTTGTTTTCATTTTT  129

Query  61   ATTTCAAGTTGGACAGATCTTGGAGATAATTTCTTACCTCACATAGATGAGAAAACTAAC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  130  ATTTCAAGTTGGACAGATCTTGGAGATAATTTCTTACCTCACATAGATGAGAAAACTAAC  189

Query  121  ACCCAGAAAGGAGAAATGATGTTATAAAAAACTCATAAGGCAAGAGCTGAGAAG  174
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  190  ACCCAGAAAGGAGAAATGATGTTATAAAAAACTCATAAGGCAAGAGCTGAGAAG  243
```

Figure 9: Blast alignment of CRP sequence in an obese subject showing the CT heterozygote

Table 2: Genotypes of USF1 gene and CRP gene observed in subjects of present study with respect to the SNPs

S.no	SNP	CC	CT	TT	Allele frequency	
1	rs3737787	20 25	5 0	-	C=0.9,T=0.1	Patients Controls
2	rs1130864	21 24	4 1	-	C=0.9,T=0.1	Patients Controls

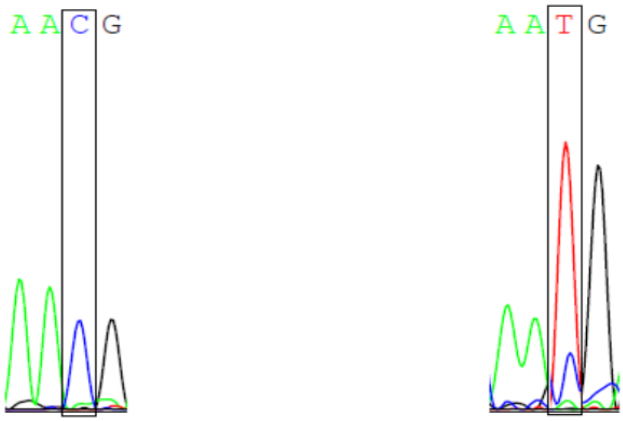

Figure 10: Homozygous CC and heterozygous CT change in CRP gene as visualized on chromatogram

DISCUSSION

Upstream transcription factor 1 (USF1) is a ubiquitously expressed transcription factor, and is a member of the basic helix-loop-helix leucine zipper family. The most commonly studied single nucleotide polymorphisms (SNPs) of USF-1 gene are rs3737787, rs2073653, rs2073655, rs2073658, rs251640, rs2516841, rs2516839, rs2774276 and rs2516837. Furthermore, rs3737787 is located in the promoter region (-789) of junctional adhesion molecule-1 (JAM1, also known as adjacent platelet F11 receptor, F11R), which was first discovered to be a surface protein on human platelets, and has been found to be associated with central obesity and systolic blood pressure in the Chinese population (Ong *et al*., 2008). Polymorphisms of the upstream transcription factor 1 (USF1) have been associated with familial combined hyperlipidemia and coronary heart disease (Pajukanta *et al*., 2004).

Obesity, age, gender, and diabetes are important factors that influence variation in blood levels of CRP. Increased serum CRP levels have been reported in subjects with obesity, metabolic syndrome, and type 2 diabetes (T2D), indicating that these individuals present a state of subclinical, low-grade inflammation that promotes the development of atherosclerosis mediated by a process of endothelial dysfunction, increasing the risk of ischemic heart disease (Hu *et al*., 2009). Several studies have reported an association between single-nucleotide polymorphisms (SNPs) in the CRP gene with variation in blood levels of CRP, or with coronary heart disease (CHD), diabetes, microangiopathic stroke, insulin resistance, metabolic syndrome, or hypertension (Szalai *et al*., 2005; Brull *et al*., 2003; Wolford *et al*., 2003).

In particular, polymorphisms in the CRP gene on chromosome 1 have consistently been associated with basal CRP levels in both men and women and with varying degrees of risk in the development of CHD (Miller, 2005). It has been shown that elevated serum CRP is a risk factor for CHD, and there is a relationship between increased serum levels of CRP with various CHD risk factors, particularly diabetes and hypertension. The effects of the SNPs on the variation in CRP levels have been reported in various populations around the world demonstrating that the effect of CRP SNPs on CRP occurs independent of ethnicity.

In conclusion, with respect to USF1 gene polymorphism rs3737787 and CRP polymorphism rs1130864 in the present study, both the controls and patients showed more or less equal distribution of genotypes and hence no genotype could be associated with coronary heart disease risk in the subjects studied. But in order to confirm the findings, a higher sample size needs to be studied.

Conflicts of interest: The authors stated that no conflicts of interest.

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International Journal of
Life Sciences
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Special Issue A 11: January 2018
UGC Approved Journal No 48951

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Pharmacological evaluation of *Parkia speciosa* Hassk. for antioxidant, anti-inflammatory, anti-diabetic and anti-microbial activities *in vitro*

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Manuscript details:

Available online on
http://www.ijlsci.in

ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

Editor: Dr. Arvind Chavhan

Cite this article as:
Ningthoujam Sonia, Myrene R Dsouza and Alisha (2018) Pharmacological evaluation of *Parkia speciosa* Hassk. for antioxidant, anti-inflammatory, anti-diabetic and anti-microbial activities *in vitro*., *Int. J. of Life Sciences*, Special Issue, A11: 49-59.

ABSTRACT

Parkia speciosa Hassk. (Petai or Stink bean, Family Fabaceae) is a traditionally consumed medicinal plant for liver disease, oedema, kidney inflamemation, diabetes and as an anthelmintic. The phytochemical analysis of seeds of *P. speciosa* in water, methanol and hydromethanolic (1:1) extracts revealed the presence of carbohydrates, amino acid, alkaloids, saponins, tannins, flavonoids, terpenoids, glycosides, xanthoproteins and phenols. In vitro antioxidant capacity by linear regression analysis was measured by assaying DPPH radical and H_2O_2 scavenging capacities. Their respective IC_{50} values were found to be 315.75 µg/ml and 166.63 µg/ml. The hydromethanolic extract gave total phenolic, total flavonoids and FRAP values of 13.54±0.0163 mg GAE/g, 5.46±0.011 mg/g QE/g and 1.9 mM $FeSO_4$ respectively. The IC_{50} values for in vitro anti-inflammatory activities were evaluated by the following assays: lipoxygenase inhibition (IC_{50} = 493.34µg µg/ml), proteinase inhibition (IC_{50} = 1142.3 µg µg/ml) and RBC membrane stabilization (IC_{50} = 67.01 µg µg/ml) at different concentrations using aspirin as control. Studies were also carried out to assess the anti-diabetic potential by assaying the ability of the plant to inhibit pancreatic lipase and amylase activities. The % inhibition at 500 µg/ml of the plant extracts were found to be 89.5% (for lipase) and 79.2% (for amylase). Antimicrobial activity of the extracts was studied against common pathogens (*S. aureus, E. coli, P. aeruginosa* and *K. pneumoniae*). The zone of inhibition was observed using well diffusion method.

Key words: Anti-inflammatory, amylase, lipase, lipoxygenase, proteinase inhibition, *P. speciosa*.

INTRODUCTION

It is estimated that up to four billion people (representing 80% of the world's population) living in the developing world rely on herbal medicinal products as a primary source of healthcare (Bandaranayake,

2006). India has probably the oldest, richest and most diverse cultural traditions in the use of medicinal plants with an estimate of over 7500 species used by several ethnic communities (AICEP 1994; Anthropological survey of India 1994). One such plant used in Southeast Asian countries like India, Malaysia, Philippines, and Indonesia (Mitsuo and Fitriyah, 2001) is *Parkia speciosa*. It can be found in rainforests, on sandy, loamy and podzolic soils.

It also grows wildly in waterlogged locations, freshwater swamp forest and on riverbanks. Seeds of *P. speciosa* can be eaten raw, cooked or roasted. It has been used as traditional medicine to treat various ailments like diabetes, cardiovascular diseases, constipation, as a carminative, kidney pain (Gmelin et al., 1981), cancer, hepatalgia, nephritis, colic ulcers and also taken as diuretic. As *P. speciosa* (both seeds and green pericarp) is used to control blood sugar level it is believed to possess anti-diabetic effect (Suvachittanont and Pothirakit, 1988). Thus, the present investigation was undertaken to understand the potential of *P. speciosa* for antioxidant, anti-inflammatory, anti-diabetic and antimicrobial activities *in vitro*.

MATERIALS AND METHODS

Collection and preparation of samples

The raw pods of *Parkia speciosa* Hassk. were collected from Ema market, Imphal, India in May 2016. The pods were deseeded, cleaned, rinsed in distilled water, sun dried and ground into a fine powder. Crushed samples were extracted using three different solvent systems: distilled water (aqueous extract), methanol (alcoholic extract) and hydroamethanolic extract (1:1 v/v). Extraction was carried out on an orbital shaker for 24 h at room temperature. Solvents were evaporated under vacuum and the resulting extracts were stored at 4 ºC.

Fluorescence analysis

Fluorescence characteristics of the powdered seed with different chemicals were observed in daylight and ultraviolet light (Chase and Pratt, 1949).

Phytochemical screening

Phytochemical examinations were carried out for all the extracts as per the standard methodology to confirm the presence of phytoconstituents (Harborne, 1991; Khandelwal, 2009).

Detection of carbohydrates

Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates as follow: *Molisch's test*: Formation of the violet ring at the junction with 2 drops of alcoholic α-naphthol solution indicates the presence of carbohydrates. *Benedict's test:* Orange red precipitate with Benedict's reagent indicates the presence of reducing sugars. *Fehling's test*: Filtrates were hydrolyzed with dil. HCl, neutralized with alkali and heated with Fehling's A and B solutions. Presence of reducing sugars was indicated by the formation of a red precipitate.

Detection of alkaloids

Extracts were dissolved individually in dilute HCl and filtered. Tests for the presence of alkaloids were as follows: *Mayer's test:* Formation of a yellow coloured precipitate with Mayer's reagent indicates the presence of alkaloids. *Wagner's test*: Formation of brown/reddish precipitate with Wagner's reagent indicates the presence of alkaloids. *Dragendroff's test*: Formation of red precipitate with Dragendroff's reagent indicates the presence of alkaloids.

Detection of saponins

Foam test: Persistence of foam after 10 mins with 0.5 ml extract and 2 ml water, shaken well indicates the presence of saponins.

Detection of glycosides

Extracts were hydrolyzed with dil. HCl, and then subjected to test for glycosides. *Modified Borntrager's test*: Extracts were treated with $FeCl_3$ solution and immersed in boiling water for about 5 min. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

Detection of phytosterols

Salkowski's test: The plant extracts were treated with $CHCl_3$ and filtered. The filtrate thus obtained was mixed with few drops of concentrated H_2SO_4, shaken and allowed to stand. The appearance of golden yellow colour was indicative of triterpenes.

Detection of flavonoids

Lead acetate test: Formation of yellow colour precipitate with the extract and a few drops of lead acetate solution indicates the presence of flavonoids.

Detection of proteins and amino acids

Xanthoproteic test: Formation of yellow colour with extract and concentrated HNO_3 indicates the presence of proteins. *Ninhydrin test:* Formation of blue colour with extract and 0.25% w/v ninhydrin reagent boiled for a few minutes indicates the presence of amino acid. *Millon's test:* A reddish-brown coloration with the extract and Millon's reagent gently heated indicates the presence of tyrosine residue.

Detection of tannins

Ferric chloride test: Formation of bluish-black colour with the extract and 3-4 drops of $FeCl_3$ indicates the presence of phenols.

Detection of terpenoids

Salkowski test: Appearance of reddish brown colour with 5 ml of extract, few drops of $CHCl_3$ and 3 ml concentrated H_2SO_4 revealed the presence of terpenoids.

Total phenolic, flavonoid and FRAP activity:

Total phenolic contents were estimated according to the spectrophotometric method using gallic acid as standard (Slinkard and Singleton, 1977) and expressed in terms of gallic acid equivalent (mg of GAE/g of tissue). Aluminum chloride colorimetric method was used for determination of total flavonoids (Chang et al., 2002) and expressed in terms of quercetin equivalent (mg of QE/g of tissue). The ferric ions (Fe^{3+}) reducing antioxidant power (FRAP) method was used to measure the reducing capacity of plant extracts (Oyaizu, 1986).

Evaluation of Antioxidant Activity

DPPH radical scavenging activity*:*

Standard ascorbic acid was pipetted out into different test tubes (100-500 µg/ml). 0.1 ml solution of each dilution was taken and made up to 3 ml with DPPH (20 µg/ml). The test tubes were incubated for 10 min at room temperature. The contents of each tube were mixed well, and the absorbance was measured at 517 nm against a blank (Braca et al., 2012). The percentage inhibition of DPPH by the samples was calculated as follows.

$$\% \text{ inhibition} = \frac{O.D. \text{ of Control} - O.D. \text{ of Sample}}{O.D. \text{ of Control}} \times 100$$

..... Eq. 1

Hydrogen peroxide scavenging activity:

Plant extract in 3.4 ml phosphate buffer was added to 0.6 ml of 45 mM H_2O_2 solution and the absorbance of the reaction mixture was recorded at 230 nm against a blank containing only buffer (Ruch et al., 1989). Ascorbic acid was used as standard. The concentration of H_2O_2 (mM) in the assay medium was determined using a standard curve ($y = 0.1223x+29.62$; $R^2 = 0.586$). H_2O_2 scavenging ability was calculated as IC_{50}. The percentage inhibition was calculated as in Eq. 1.

Evaluation of *In vitro* Anti – Inflammatory Activity

Lipoxygenase inhibition:

Soybean lipoxygenase activity was assayed (Axelrod et al., 1981). The reaction contained 2.9 ml 0.1M borate buffer pH 9.0 and 50 µl 10 mM linoleic acid. The reaction was initiated by the addition of 50 µl of the soybean enzyme extract. The enzyme activity was measured by following the formation of the product, 12-HETE at 234 nm for up to 1 min. The enzyme inhibition was determined by pre-incubating the enzyme with the plant extract or standard phytochemicals prior to determining its 12-LOX activity. The percentage inhibition was calculated as in Eq. 1. IC_{50} was calculated from $y=0.0839x+8.61$; $R^2=0.788$.

Proteinase Inhibitory Action*:*

The test was performed according to the modified method of Sakat et al., (2010). 2 ml of the reaction mixture contains 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4) and 1 ml test sample of different concentrations (100 - 500 µg/ml). The mixture was incubated at 37 ^oC for 5 min and then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min. 2 ml of 70% perchloric acid was added to arrest the reaction. Cloudy suspension was centrifuged, and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in duplicate. The percentage inhibition of protein denaturation was calculated using Eq. 1. IC_{50} was calculated from $y=0.0399x+4.428$, $R^2=0.8585$.

RBC membrane stabilization activity:

Various concentrations of extract, reference sample and control were separately mixed with 1 ml of phosphate buffer, 2 ml of hyposaline and 0.5 ml of hRBC suspension. Diclofenac sodium (100 µg/ml) was used as a standard drug. All the assay mixtures were incubated at 37 ^oC for 30 min and centrifuged at 3000 rpm. The supernatant was used to estimate the haemoglobin content using a spectrophotometer at 560 nm (Azeem et al., 2010). The percentage of hemolysis was estimated assuming that the control produced 100% haemolysis.

The percentage inhibition of protein denaturation was calculated using Eq. 1. IC_{50} was calculated from y=0.1525x+39.78, R^2= 0.5502.

***In-vitro* anti-diabetic evaluation**

Alpha amylase inhibition assay:

The procedure described by Shai et al., (2010) with slight modifications was used to determine the α-amylase inhibitory activity of the fractions. A volume of 250 µl of each fraction or acarbose at different concentrations (100-500 µg/ml) was incubated with 500 µl of porcine pancreatic amylase (2 U/ml) in phosphate buffer (100 mM, pH 6.8) at 37 °C for 20 min. Thereafter, 250 µl of 1% starch dissolved in 100 mM phosphate buffer (pH 6.8) was further added to the reaction mixture and incubated at 37° C for 1 h. Dinitrosalicylate colour reagent (1 ml) was then added and boiled for 10 min. The absorbance of the resulting mixture was read at 540 nm the inhibitory activities of the fractions on α-amylase were calculated by using the following formula:

$$\% \text{ inhibition} = 1 - \frac{B-b}{A-a} \times 100 \quad \text{----- eq. (2)}$$

where 'A' is the activity of the enzyme without inhibitor, 'a' is the negative control without the inhibitor, 'B' is the activity of the enzyme with inhibitor, and 'b' is the negative control with inhibitor.

Porcine pancreatic lipase (PPL) inhibition assay: This was performed as described by Bustanji et al., (2011) with minor modification. The enzyme solution was prepared immediately before use, by suspending crude porcine pancreatic lipase powder in 0.1 M phosphate buffer (pH 7.6) (100 U/ml). The solution was then centrifuged at 2000 rpm for 10 min and the clear supernatant was recovered. Triolein (1% v/v) was used as the substrate for PLL. The plant extract (5, 12.5, 25, 100, 125, 250, 500 µg/ml) was preincubated with 200 µl of PPL solution for 5 min at 37 °C, before the addition of 800 µl triolein substrate solution. The absorbance was measured at 450 nm against blank using denatured enzyme in an ELISA reader. The denatured enzyme was prepared by boiling the enzyme solution for 5 min. Orlistat was used as a reference drug. The extract was dissolved in DMSO at a final concentration not exceeding 1% (v/v). The activity of the negative control was checked in the presence and absence of the inhibitor. The % inhibition was calculated according to the formula (2).

Mechanism of porcine pancreatic lipase inhibition: The inhibition mode of crude extract of *P. speciosa* on porcine pancreatic lipase (PPL) was assayed with increasing concentrations of the substrate Triolein (20, 40, 60, and 80 µM) in the presence and absence of two different concentrations of the extracts (100 and 200 µg/mL). The mode of inhibition was determined by Lineweaver-Burk plot.

Screening for Antibacterial activity of methanolic extract

Agar well diffusion assay:

The antibacterial activity was carried out by employing 24 h cultures of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae.* The standard medium Mueller Hinton Agar, was poured to a depth of 4 mm in a 90 mm petridish. The bacterial inoculum was prepared from an 18 h broth culture of the microbe to be tested and was standardized with sterile physiologic saline to contain 106 cfu/ml. A well (6 mm diameter) was made using a sterile cork borer. The standard drug and extracts were placed in the well. Antibacterial assay plates were for overnight incubation. Ciprofloxacin (5 µg/disc) was used as a positive control for antibacterial activity. After 24 h of incubation at 37 ^{o}C, zone of inhibition (ZOI) was observed and diameter measured.

Statistical analysis:

Data of *in vitro* assays recorded were analyzed using Microsoft Excel to determine IC_{50}. One-way analysis of variance (ANOVA) was performed and $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

Folkloric medicine from natural plant products has traditionally been used to cure a number of common ailments as they are a rich source of bioactive molecules. More intensive research involving the discovery of anti-inflammatory and anti-diabetic agents from medicinal plant sources is currently underway because of its known ability of suppressing relevant reactions of the inflammatory cascade. The present study was carried out to assess the validity of ethanomedicinal use of *P. speciosa* in the management and treatment of inflammatory disorders.

Table 1: Fluorescence analysis of pod powder of *P. speciosa.*

Sl No.	Experiment	Visible/Day light	UV light (365nm)
1	Powder as such	Light green	Light green
2	Powder+1N HCL	Colourless	Light green
3	Powder+Conc.H_2SO_4	Dark brown	Yellowish green
4	Powder+50% H_2SO_4	Dark brown	Fluorescent brown
5	Powder+ Conc.HNO_3	Yellow	Dark green
6	Powder+ Conc.HCl	Light yellow	Yellowish green
7	Powder+50%HNO_3	Yellow	Dark green
8	Powder+ Acetic acid	Light green	Yellow
9	Powder+ Ferric chloride	Light brown	Brownish green
10	Powder+HNO_3+NH_3	Light yellow	Green
11	Powder+ NH_3	Transparent	Light green
12	Powder+ Benzene	Light yellow	Orange
13	Powder+ Petroleum ether	Light green	Fluorescent orange
14	Powder+ Acetone	Green	Fluorescent orange
15	Powder+ Chloroform	Light green	Reddish orange
16	Powder+ Methanol	Light green	Fluorescent orange
17	Powder+ Ethanol	Light green	Fluorescent pink

Table 2: Phytochemical investigation of *P. speciosa* seed extract

Sl. No.	Phytochemical analyzed	Test performed	Result		
			80% methanol	Methanol:water (1:1)	Aqueous
1	Alkaloid test	Mayer's test	+	++	+
		Wagner's test	+	++	+
		Dragendoroff's test	+	++	+
2	Carbohydrate test	Molish's test	+	++	+
		Benedict's test	+	++	+
		Fehling's test	+	++	+
3	Saponin test	Foam test	+	++	+
4	Glycosides test	Borntrager's test	+	++	+
5	Steroid test	Salkowaski test	-	+++	+
6	Flavonoid test	Lead acetate test	+	+++	+
7	Proteins and amino acids	Ninhydrin test	+	++	+
		Xanthoproteic test	+	++	+
		Millon's test	+	++	+
8	Tannins test	Ferric chloride test	+	++	+
9	Terpenoids test	Salkowski test	+	++	+

- denotes absence, + denotes presence, + + denotes average, +++ denotes abundance of phytochemicals

Table 3: Quantitative analysis of total phenols, total flavonoids, FRAP activity

Sample	Total Phenols (mg GAE/g DW)	Total Flavonoids (mg QE/g DW)	FRAP activity (mM $FeSO_4$)
P. speciosa	13.54 ± 0.016	5.46 ± 0.011	1.9

All values are represented as mean±SD (n=3). GAE – Gallic acid equivalents; QE – Quercetin equivalents.

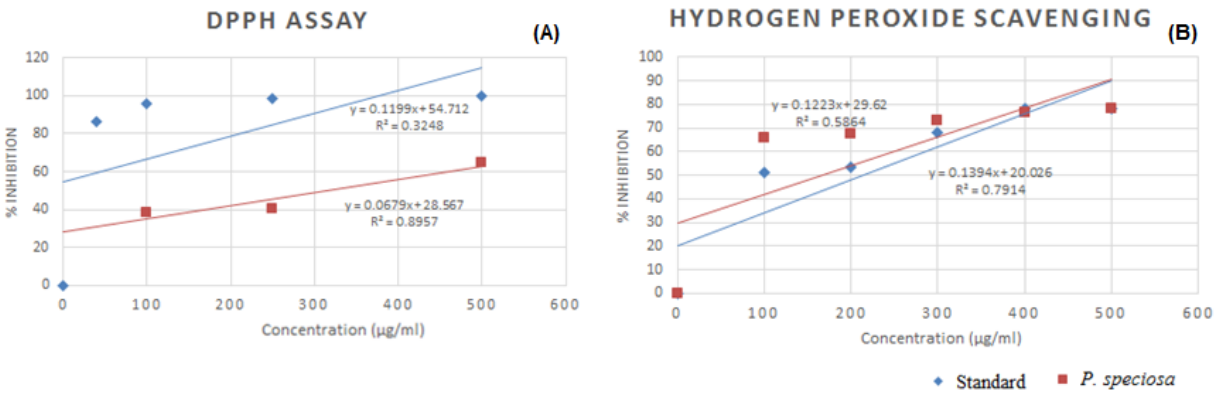

Figure1: Antioxidant activities of seed of *P. speciosa*; DPPH assay (A), H_2O_2 scavenging activity (B)

Fluorescence analysis:

The pods of *P. speciosa* produced different colours and fluorescence under UV light and day light when treated with various reagents (Table 1).

Phytochemical screening:

Phytochemicals or phytoconstituents are defined as secondary metabolites which are produced by majority of plants possessing medicinal uses. The analysis of revealed the presence of saponins, flavonoids, terpenoids, coumarins, quinines, cardiac glycosides, xanthoproteins, steroids, phenols, carboxylic acid group alkaloids, tannins, terpenoids, glycosides, resins in varying concentrations (Table 2).

Total phenols and total flavonoid content:

Secondary metabolites such as phenols are synthesized by the pentose phosphate, shikimate, and phenylpropanoid pathways in plants (Randhir et al., 2004). Flavonoids, the most common of these are known to provide health benefits through their antioxidant activity and modulation of cell signalling pathways. The total phenolic and flavonoid contents of hydromethanolic extract of *P. speciosa* was found to be 13.54±0.016 mg GAE/g and 5.46±0.011 mg/g QE/g of fresh weight tissue respectively (Table 3). The consumption of *P. speciosa* could be attributed to the wide range of physiological properties it exhibits, such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects (Benavente-Garcia et al., 1997; Manach et al., 2005; Middleton et al., 2000).

***In vitro* antioxidant activity**

The antioxidative activities observed in plant extracts are attributed to the different synergistic mechanisms exhibited by various polyphenolic compounds acting as free radical scavengers due to their redox properties. They play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Udegbunam et al.,2010).

FRAP assay:

Fe^{3+} reducing antioxidant power assay has been used to determine antioxidant activity as it is simple and quick and further contributed by the reproducibility of the reaction and the linear relation to molar concentration of the antioxidants. The results showed that FRAP values were higher in hydromethanolic extracts of the plants when compared to the standard ascorbic acid (Table 3).

DPPH scavenging activity:

Total antioxidant capacity of the extracts was calculated using % inhibition against concentration of ascorbic acid (y=0.1199x+54.71; R^2=0.325) (Fig 1A). At 500 µg/ml concentration, *P. speciosa* extract exhibited DPPH radical scavenging activity that was found to be 64.52±2.4 µg/ml. The IC_{50} from the graph were found to be 194.52 µg/ml (ascorbic acid, y = 0.1199x+54.712, R^2=0.3248) and 315.75 µg/ml (*P. speciosa,* 0.0679x+28.56, R^2 =0.6225) (Table 4). The results of the present investigation demonstrate that *P. speciosa* can significantly decrease *in vitro* DPPH radical concentration, thus suggesting that plant extract contains secondary metabolites with strong antioxidant activity.

H_2O_2 scavenging activity:

H_2O_2 crosses cell membranes rapidly, reacts with Fe^{2+} and Cu^{2+} ions to form hydroxyl radicals thereby leading to the generation of toxic effects (Wagner et al.,1996; Handa et al., 2006). H_2O_2 is a weak oxidizing agent and

can inactivate few enzymes and proteins directly, usually by oxidation of essential thiol (-SH) groups. Scavenging of hydrogen peroxide and its percentage inhibition in methanolic extracts of seeds of *P. speciosa* demonstrated IC_{50} values of 166.63 µg/ml (y=0.1223x+29.62; R^2=0.586) as given in the Table 4. Ascorbic acid taken as reference showed an IC_{50} value of 215.68 µg/ml (y=0.1394x+20.0; R^2= 0.7914) (Fig 1B).

Table 4: *In vitro* antioxidant and anti-inflammatory activity of *P. speciosa* seed extract

	Assay	Control			*P. speciosa*	
		Conc. (µg/ml)	% inhibition	IC_{50} (µg/ml)	% inhibition	IC_{50} (µg/ml)
***In vitro* antioxidant activity**	**DPPH scavenging activity**	100	28.6 ± 9.2	194.52	20.3 ± 1.1	315.75
		200	64.35 ±2.1		38.7 ± 7.8	
		300	64.20 ±9.8		40.19 ±6.0	
		400	72.34±2.9		55.31±3.8	
		500	80.83 ±3.4		64.52 ±2.4	
	H_2O_2 scavenging activity	100	51.01± 3.4	215.68	65.91± 3.9	166.63
		200	53.68±2.8		67.53± 7.6	
		300	67.93± 9.9		72.97± 2.3	
		400	78.21± 7.4		76.70± 6.6	
		500	78.42± 5.6		78.06± 5.7	
***In vitro* anti-inflammatory activity**	**LOX inhibiting activity**	100	45.14± 7.8	280.71	25.73± 5.5	493.34
		200	48.26± 8.3		30.24± 1.1	
		300	51.1± 10.2		32.30± 4.7	
		400	56.9± 11.4		38.6± 10.2	
	Proteinase inhibitory activity	100	3.15± 0.3	899.1	10.89± 2.4	1142.3
		200	6.01± 1.3		16.07± 5.7	
		300	10.7± 2.7		17.73± 3.1	
		400	21.3± 4.8		18.86± 1.1	
		500	29.9± 5.9		22.78± 3.6	
	RBC membrane stabilization	100	88.21± 11.2	53.75	86.54± 8.1	67.01
		200	95.26± 8.9		89.61± 10.3	
		300	96.67± 9.6		94.63± 9.8	
		400	97.44± 12.1		97.45± 6.7	
		500	99.36± 6.7		99.21± 12.6	
***In vitro* anti-diabetic activity**	**Amylase inhibitory action**	100	18.9 ± 2.4	324.18	36.84 ± 4.8	199.29
		200	30.3 ± 3.7		48.22 ± 11.1	
		300	54.2 ± 6.9		56.06 ± 9.6	
		400	69.8 ± 10.1		69.8 ± 4.5	
		500	79.2 ± 12.2		79.2 ± 9.6	
	Pancreatic lipase inhibitory action	25	68.1 ± 4.5	227.27	72.9 ± 3.5	196.61
		100	71.9 ± 3.2		72.9 ± 4.7	
		125	73.7 ± 8.9		81.2 ± 9.6	
		250	76.1 ± 9.2		88.5 ± 7.1	
		500	76.3 ± 8.6		89.5 ± 5.9	

Table 5: Kinetic analysis of pancreatic lipase inhibition by crude extracts of *P. speciosa*

	Velocity of enzyme activity at different concentration of substrate [S] (µM)				Vmax (µM min^{-1})	Km (µM)
	20	40	60	80		
Control	2.71	3.968	10.85	12.345	333.33	2595.34
P. speciosa	1.2	2.967	3.636	5.000	44.05	738.85

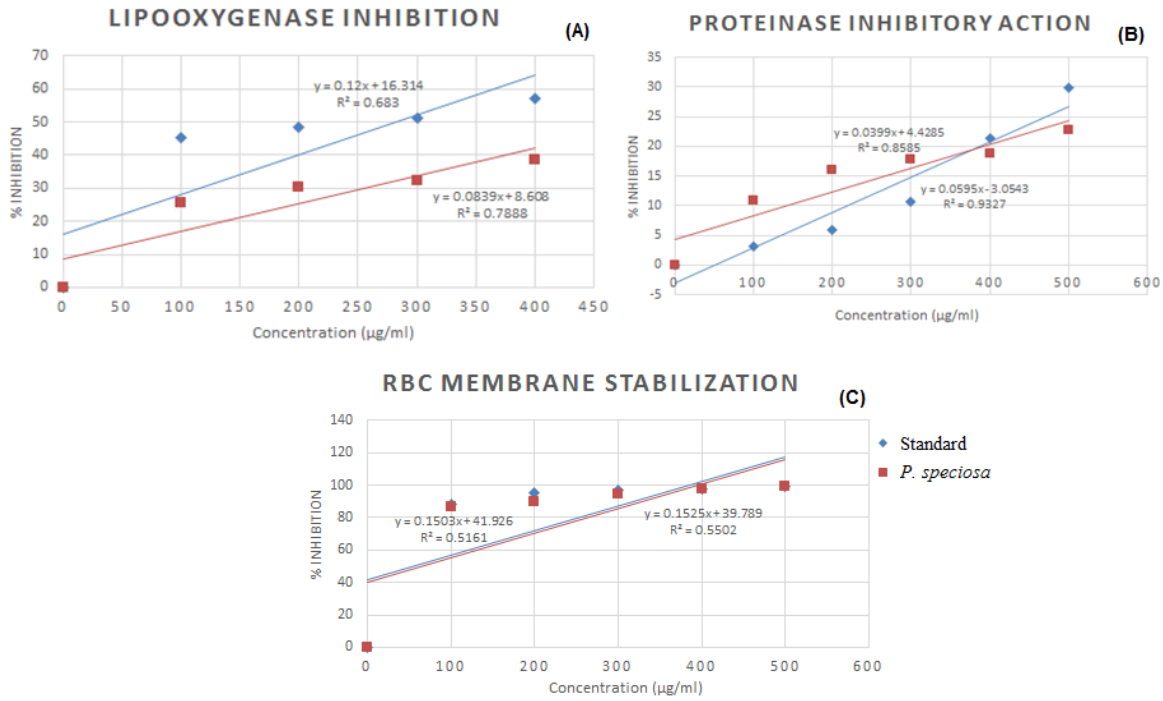

Figure 2: Anti-inflammatory activities of seed extract of *P. speciosa*; lipoxygenase inhibition assay (A), proteinase inhibitory action (B), RBC membrane stabilization assay (C).

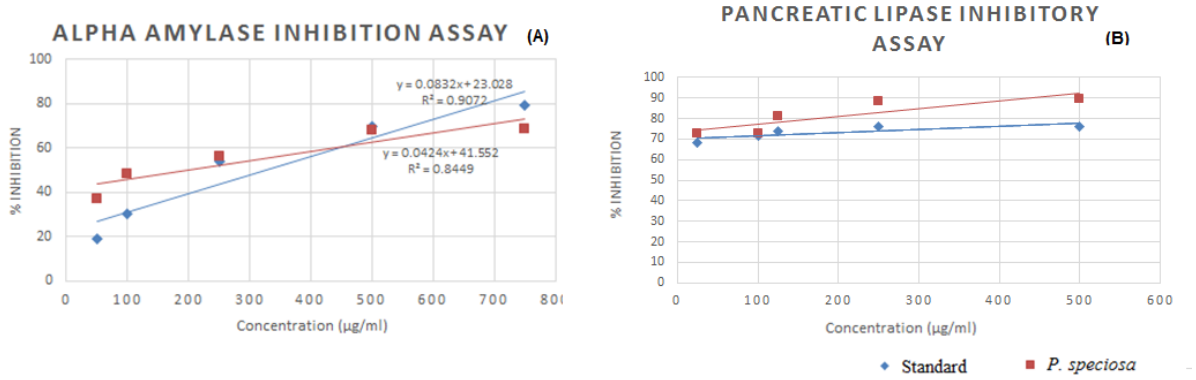

Figure 3: Anti-diabetic activities of seed extract of *P. speciosa*; alpha amylase inhibition assay (A), pancreatic lipase inhibitory assay (B).

***In vitro* anti-inflammatory activity**

Inhibition of Lipooxygenase activity:

Lipoxygenases (LOXs) comprise a family of non-heme iron-containing dioxygenases, representing the key enzymes in the biosynthesis of leukotrienes that have been postulated to play an important role in the pathophysiology of several inflammatory and allergic diseases (Ghosh,1998). In general, lipoxygenase inhibitors can bind covalently to iron or form the molecular complexes blocking access to iron (Skrzypczak-Jankun et al., 2007). The plant lipoxygenase pathway is in many respects equivalent to the 'arachidonic acid cascades' in animals. For this reason, the *in vitro* inhibition of lipoxygenase constitutes a good model for the screening of plants with anti-inflammatory potential (Abad et al., 1995). Methanolic extracts of seed of *P. speciosa* were studied at 100-500 µg/ml, and the inhibition obtained is as shown in Table 4. From these results, the strongest inhibition for all the samples was obtained at concentration of 400 µg/ml. The standard exhibited 56.9±11.4 % inhibition at a concentration of 400 µg/ml. Percentage inhibition of lipoxygenase in methanolic extracts of seed *P. speciosa* demonstrated IC_{50} values of 493.34 µg/ml (y=0.0839x+8.61, R^2=0.788) (Table 4, Fig 2A). Ascorbic acid taken as reference exhibited 280.71 µg/ml (y=0.12x+16.31, R^2 = 0.683).

Proteinase Inhibitory activity:

Activated leukocytes are widely implicated in cardio-vascular disease (CVD). Mononuclear cells are recruited to sites of vascular injury thus contributing to foam cells within atherosclerotic plaques (Mandal, 2013). Activated white blood cells discharge into the surrounding milieu reactive oxygen species (ROS) and a variety of proteolytic enzymes, particularly serine proteases (Venkatalakshmi et al., 2015). Inhibition of 22.78% and 29.9% at 500µg/ml was observed for *P. speciosa* and aspirin respectively (Table 4). The IC_{50} values as calculated from graph were found to be 899.1 µg/ml (Aspirin, y=0.0595x+3.054, R^2=0.937), 1142.3µg/ml (*P. speciosa,* y=0.0399x+4.428, R^2=0.858) (Fig 2B).

RBC membrane stabilization activity:

The erythrocyte plasma membrane resemblances to the lysosomal membrane and hence the stabilizing effect of drugs on erythrocyte membrane may correlate with its lysosomal membrane stabilizing effect (Debnath et al.,2013). The lysosomal membrane stabilization leads to the inhibition of release of the inflammatory mediators and consequent inhibition of the process of inflammation (Bhutkar and Bhise, 2012). Maximum inhibition was observed at 500 µg/ml, where aspirin and *P. speciosa* showed 99.36 and 99.21% inhibition respectively (Table 4). The IC_{50} values for the standard drug and *P. speciosa* was found to be 53.75 µg/ml (y=0.1503x+41.9, R^2=0.516) and 67.01 µg/ml (y=0.1525x+39.79, R^2=0.5502) (Fig 2C).

***In vitro* anti-diabetic activity**

Alpha amylase inhibition assay:

The high prevalence of diabetes as well as its long-term complications has led to an ongoing search for hypoglycaemic agents; over the years, various medicinal plants and their extracts have been reported to be effective in the treatment of (Debnath et al., 2013). The digestive alpha amylase is responsible for hydrolyzing dietary starch (maltose), which breaks down into glucose prior to absorption. Inhibition of alpha amylase can lead to reduction in post prandial hyperglycemia in diabetic condition. The results showed that extracts of *P. speciosa* have antidiabetic activity which is compared with acarbose standard. *P. speciosa* showed a maximum inhibition of 79.2% at 500 µg/ml (Table 4). The IC_{50} of *P. speciosa* and standard acarbose were found to be 199.29 µg/ml (y=0.04242x+41.55, R^2=0.8449) and 324.18 µg/ml (y=0.0832x+23.02, R^2=0.9072) (Fig 3A). This shows that *P. speciosa* has anti-diabetic activity.

Pancreatic lipase (PPL) inhibitory assay:

Pancreatic lipase is a key enzyme in dietary triacylglycerol absorption, hydrolyzing triacylglycerol to 2-monoacylglycerol and fatty acids. It is well known that dietary fat is not directly absorbed from the intestine unless it has been subjected to the action of pancreatic lipase (Skrzypczak-Jankun et al., 2007). The polyphenolic extracts from a number of plants have been shown to be effective inhibitors of the intestinal pancreatic lipase enzyme (Harborne, 1998). A dose-dependent manner of inhibition was exhibited by the plants extract (Table 4). Maximum *in vitro* inhibitory activity exhibited by *P. speciosa* and Orlistat, the standard drug and pancreatic lipase inhibitor was found to be 89.5% and 76.3 % at 500 µg/ml respectively. The IC_{50} of *P. speciosa* and standard Orlistat were found to be 227.27 µg/ml and 196.61 µg/ml (Fig 3B).

Kinetic study of pancreatic lipase:

The mode of inhibition of the enzyme pancreatic lipase by *P. speciosa* was studied by double-reciprocal Lineweaver-Burk at 100 µg/ml as shown in Fig 4. Since both the kinetic parameters i.e., the maximal velocity of the PPL enzyme-substrate extract reaction (V_{max}) and the affinity (K_m) as determined from the double reciprocal trend lines were affected by the extract concentration, a mixed mode of inhibition was predicted. The Michaelis-Menten parameters are tabulated in Table 5. The mixed mode inhibition exhibited by pancreatic lipase means that the inhibitor binds to the enzyme whether or not the enzyme has already bound the substrate but has a greater affinity for one state or the other, thus resulting in the increase of K_m.

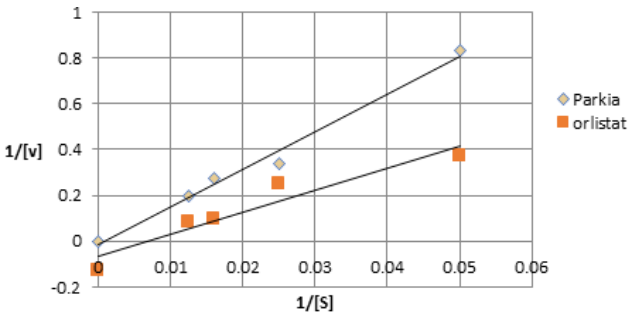

Figure 4: Lineweaver-Burke plot of *P. speciose* extract against Pancreatic lipase

Antimicrobial Activity of Extracts:

Most of the bioactive medicinal metabolites are synthesized via plant secondary metabolic pathways during the vegetative stage of a plant's life cycle and

Table 6: Antibacterial activity of extracts of *P. speciosa*. The zone of inhibition is expressed in mm

Strain	Zone of Inhibition (mm)	
	P. speciosa	**Ciprofloxacin**
E. coli	6	7
K. pneumoniae	8	11
P. aeruginosa	9	11
S. aureus	10	20

these compounds are responsible for their therapeutic properties. In general, these compounds effectively inhibit and/or stop microbial growth via disruption of the synthesis of microbial nucleic acids, proteins and cell walls (Randhir et al., 2004). It was found that the methanolic extract of *P. speciosa* showed highest zone of inhibition for the gram positive *S. aureus* at 10 mm (Table 6).

CONCLUSION

The present study was carried out to assess the potential of *P. speciosa* Hassk. as an alternative to synthetic drugs. The hydro-alcoholic extract possesses potential antioxidant, anti-inflammatory, anti-diabetic and anti-microbial activities. The presence of high levels of phenolics and flavonoids in the sample could be responsible for its radical scavenging activities. In addition, its use in ethanomedicine has been substantiated by its ability to inhibit pancreatic amylase, pancreatic lipase, lipooxygenase and proteinase enzymes. There arises further need to explore the bioactive constituents responsible for this activity as well as to elucidate the exact mechanism of action and to extrapolate the results on animal models in order to establish possible side effects. Thus, further understanding of metabolic engineering and applying its principle to enhance the synthesis and accumulation of bioactive compounds is the need of the hour.

Acknowledgement

The authors are grateful to Mount Carmel College, Autonomous, Bengaluru, India for provision of facilities needed for conducting this project.

Conflicts of interest: The authors stated that no conflicts of interest.

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SJIF Impact Factor-4.115
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Published in: February, April, June, August, October & December

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Special Issue A 11: January 2018
UGC Approved Journal No 48951

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Hepatoprotective and antidiabetic effect of aqueous extract of *Costus spicatus* jacq. Rhizome extract in streptozotocin induced diabetic Rats –histological study

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Manuscript details:

Available online on
http://www.ijlsci.in

ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

Editor: Dr. Arvind Chavhan

Cite this article as:
Azhagumadhavan S, Vinayagamoorthi P, Kavitha P and Ganesan S (2018) Hepatoprotective and antidiabetic effect of aqueous extract of *Costus spicatus* jacq. Rhizome extract in streptozotocin induced diabetic Rats –histological study, *Int. J. of. Life Sciences*, Special Issue, A11: 61-66.

ABSTRACT

Diabetes mellitus is an endocrine, metabolic disorder in which the homeostasis of carbohydrate and lipid metabolism is improperly regulated by the pancreatic hormone, insulin, ultimately resulting in increased blood glucose. In our study, diabetes was induced in rats by single intraperitoneal injection of Streptozotocin (STZ) at a dose of 45mg/kgb.w. and the hepatoprotective efficacy of aqueous extract of Costus spicatus (ACS) at a dose of 500 mg/kg b.w. was studied. Diabetes mellitus is associated with progressive metabolic derangement, worsening glycemic control, and morphological changes in the liver, pancreas and other organs. Diabetes is characterized by high blood plasma glucose levels and high concentration of liver enzymes like SGOT, SGPT and ALP in hepatocytes of the liver leaks into the circulation as a result of damage to cell membrane of hepatocytes. In the present study, administration of ACS to STZ induced diabetic animals restored the blood plasma glucose and liver enzymes to normal level which is comparable to the anti-diabetic efficacy of Glibenclamide a synthetic drug. Though both showed its anti-diabetic efficacy, ACS exerted its anti-diabetic activity without damaging the liver in comparison with Glibenclamide which damaged the liver.

Keywords: Hepatoprotective, Insulin, Diabetes mellitus, Pancreatic hormone, STZ.

INTRODUCTION

Diabetes mellitus (DM) has shown an exponential rise of causing serious economic, social and health repercussions. Diabetic retinopathy (DR), the most common and serious complication of DM, are characterized by vascular alterations including retinal blood flows changes, endothelial cells dysfunction, breakdown of the blood-retinal barrier, ischemia and neovascularisation. DR has no or mild symptoms at early stages; however, if not

properly treated, DR may progress to the advanced stage, during which severe pathologies often lead to irreversible blindness (Cheung *et al*., 2010; Antonetti *et al*., 2012; RaskpMadsen *et al*., 2013).

Diabetes is becoming the third killer disease of mankind, after cancer and cardiovascular disease, because of its high prevalence, morbidity and mortality (Li *et al*., 2004). Diabetes is the fastest growing chronic disease in the world; the number of diabetic patients is increasing, almost half of all deaths before the age of 70 have been attributed to high blood glucose levels associated with diabetes (WHO, 2016). Diabetes is being treated using synthetic drugs to achieve euglycemia and eliminating or minimizing the chronic complications as well. But the usage of synthetic anti-diabetic drugs is associated with lot of side effects (Holman 1991).

Herbal medicines have received more attention than synthetic drugs for the treatment of various human diseases including diabetes due to their less or nil side effects and cost effectiveness (Wachtel-Galor 2011). Nowadays, treating diabetes using medicnal herbs gaining its ground at a fast pace. However, the mechanism of action of anti-diabetic activity of medicinal herbs has not yet been clearly established. It has been proposed that the hypoglycemic effect of these medicinal herbs is due to their ability to restore or stimulate the function of pancreatic tissues or β-cells thereby causing an increase in insulin level or inhibition of intestinal absorption of glucose.

Costus igneus is also known as fiery *costus* or spiral flag or insulin plant belongs to the *costaceae* family, contains a range of phytochemicals which include flavonoids, alkaloids, terpenoids and it is traditionally being used in India to control diabetes (Devi *et al*., 2008; Saraswathi *et al*., 2010; Bhat *et al*., 2010; Shetty *et al*., 2010 a,b; Krishnan *et al*., 2011; Pazhanichamy *et al*., 2011). The present study aimed to elucidate the anti-diabetic activity of *Costus igneus* and the mechanism by which it exerts its anti-diabetic activity in Streptozotocin induced diabetes rat models.

MATERIALS AND METHODS

Animal

Albino wistar male rats; 10- weeks old with a body weight ranged between 180-250 g were used. Animals were housed under standard conditions temperature (24±2°C) and relative humidity (30-70%) with a 12:12 (light:dark) conditions. The animals were fed with standard pellet diet. Animals were handled according to Good Laboratory Practice. Ethical clearance was obtained from Institutional Animal Ethics Committee and all experiments were conducted according to the Indian National Science Academy guidelines for the use and care of the experimental animals.

Plant collection and extraction

Costus spicatus were collected from Saliyamangalam, Thanjavur District, Tamil Nadu, India. Rhizome were cut into small pieces and shade dried at room temperature. The dried rhizome was subjected to size reduction to a coarse powder by using dry grinder and sieved. About 100 g was continuously extracted with ethanol (95%) using Soxhlet extractor up to 48 h. The extract was filtered through Whatmann filter paper and concentrated using rotary evaporator at 40-60ºC under reduced pressure to prepare final crude extract.

Diabetes induction using Streptozotocin

Animals were fasted overnight and diabetes was induced by single intra peritoneal injection of Streptozotocin (STZ) (45mg/kg body weight) prepared in 0.1 M Citrate buffer at pH 4.5. To overcome drug induced hypoglycemia, animals were allowed to drink 5% glucose solution overnight. Citrate buffer in place of Streptozotocin was injected to control rats. After 72 hours of STZ injection, (taken as 0^{th} day) fasting blood glucose levels of each animal was analyzed. Animals with fasting blood glucose levels > 200 mg/dl were considered as diabetic and considered for the study.

Anti-diabetic treatment of animals

The rats were randomly divided into 5 groups and each group consisted of 6 rats and the duration of treatment was 45 days. Group I: Animals fed with distilled water (Negative control). Group II: Diabetic animals fed with distilled water (positive control). Group III: Diabetic animals fed with Glibenclamide (5mg/kg/b.w./day). Group IV: Non-diabetic animals fed with ACS (500 mg/kgb.w./day). Group V: Diabetic animals fed with ACS (500mg/kg/b.w./day). Before (0^{th}), during (21^{st}) and at the end of treatment (45^{th}), body weight, fasting plasma glucose levels and serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and alkaline phosphatase (ALP) levels were measured. Plasma glucose levels were determined by Ortho Toludine reagent method. SGOT, SGPT and ALP levels were measured from serum separated from blood

which was collected from the retro-orbital plexuses of the rats of all groups under light ether anaesthesia using a semiautomatic biochemical analyzer with commercially available biochemical kits.

Collection of tissue samples and histological analysis

After 45 days of treatment animals were sacrificed following the guidelines of animal ethical committee. The liver tissues were excised and fixed in 10% neutral buffered formalin (NBF). Thus fixed liver tissues were sectioned with Leica rotary microtome to produce serial sections of 5µ thickness. Liver sections were stained with Hematoxylin and Eosin (H&E) stains. The stained specimens were then analyzed and photomicrographed with APCAM-5 USB 2digital camera attached to a computer monitor (ADELTAVISION OPTEC India microscope Ltd).

Statistical Analysis

One-way analysis of variance (ANOVA) was performed using the software "Graphpad Instat". Results were expressed as mean ± SEM. $p<0.05$ was considered as statistically significant.

RESULTS

The administration of STZ resulted in a significant increase in plasma glucose level, SGOT, SGPT and ALP along with a reduction in body weight (Table 1, 2 & 3). After treatment of animals with 500 mg/kg/ b.w of ACS, the plasma glucose levels significantly reduced and returned to normalcy ($p<0.001$), with simultaneous increase in body weight (Table 1 & 2).

Table 1: Effect of ACS on body weight in normal & STZ induced diabetic rats

Groups	Change in Body weight (gm)		
	0 day	21st day	45 st day
Group I	167±2.58	86.66±2.41	197.16±2.98
Group II	185.66±2.13**	164.83±1.47**	127.33±1.96**
Group III	174.33±2.15#	170.33±2.44**	189.16±1.97**
Group IV	178.16±1.60	186.50±2.14**	180.66±1.60**
Group V	195±2.78#	183.66±2.21**	185.50±1.45**

*Results are expressed as mean ±SEM; n=6; **=p<0.001 and# =not significant*

Table 2: Effect of ACS on plasma glucose values in normal & STZ induced diabetic rats

Groups	Change in Body weight (gm)		
	0 day	21st day	45 st day
Group I	99.16±2.12	97.33±1.76	96.5±2.12
Group II	279.33±8.80**	339±11.07**	379.83±11.85**
Group III	266.66±8.53#	197±7.10**	123.5±2.95**
Group IV	89.70±0.76	89.5±1.47**	89.16±0.70**
Group V	267.50±7.02#	199.5±10.67**	127.5±2.39**

Results are expressed as mean ±SEM;n=6; **=p<0.001 and # =not significant.

Table 3: Effect of ACS on SGOT, SGPT and ALP levels in normal & STZ induced diabetic rats

Groups	SGOT (IU/L)		SGPT (IU/L)		ALP (IU/L)	
	0 day	45 th day	0 day	45 th day	0 day	45 th day
Group I	65.01±3.40	62.58±1.43	77.33±0.66	77±1.34	77±1.24	79.33±0.82
Group II	154.45±2.64**	225±3.50*	249.87±2.05**	147.57±6.67**	144.68±1.57**	209±1.17**
Group III	142.37±1.67#	103±1.28**	97.86±1.07**	145.33±1.89#	146.33±1.78#	95.4±1.53
Group IV	46.25±0.65	61.33±0.63**	67.89±55.3**	78.85±0.08	78.87±0.70	79.3±0.99**
Group V	141.37±3.77#	177±1.94#	76±2.17**	145.66±2.37#	147.60±2.34#	81.17±0.85**

Results are expressed as mean ± SEM; n=6; ** =p<0.001 and # = not significant

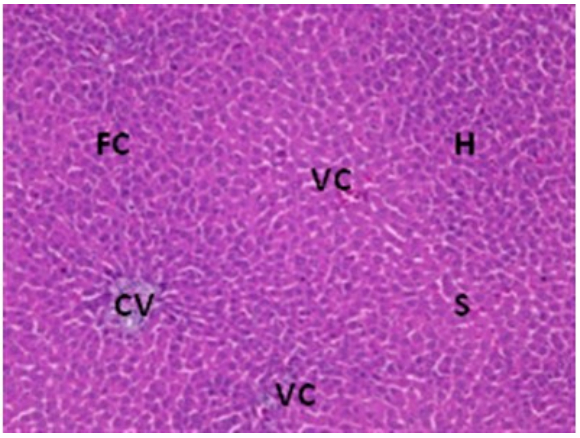
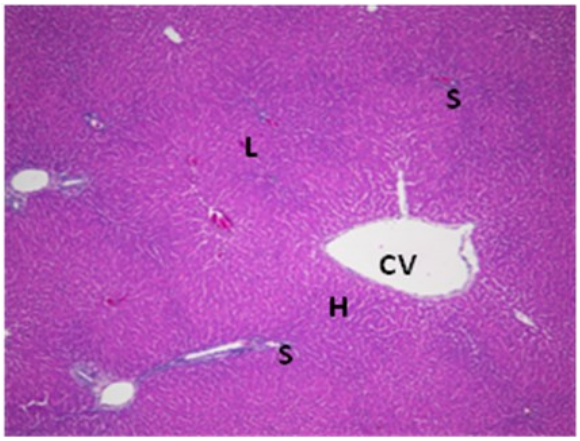


Figure (1A)



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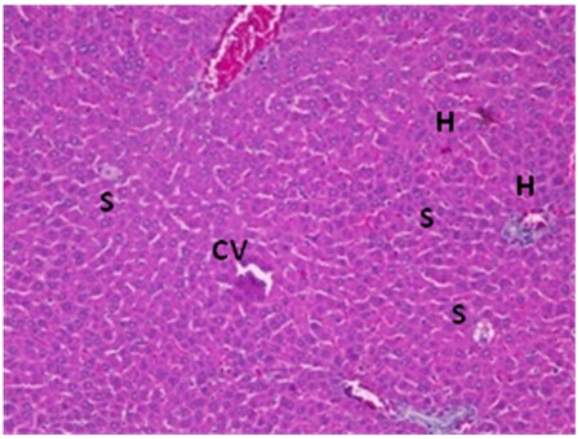

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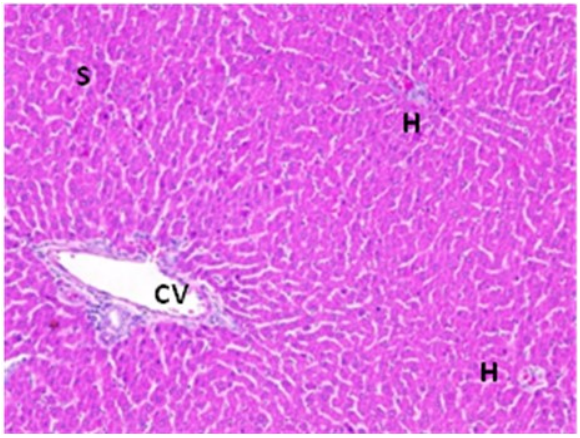

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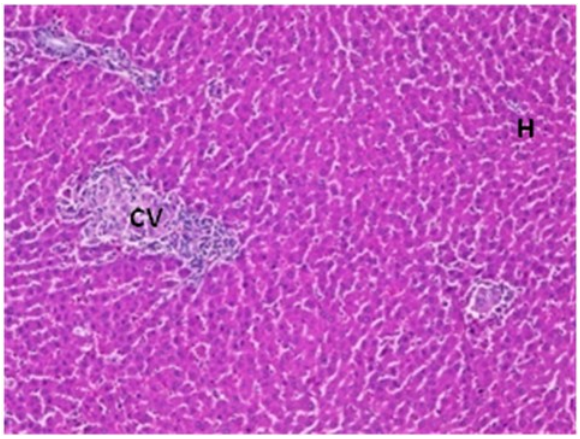

Figure (1E)

Cv-Central Vein ; **Vc**-Vacuolation ; **Fc-**Fatty Changes ; **H**-Hepatocyte ; **S-** Sinusoids

Figure 1A: Photomicrograph of liver of STZ induced diabetic rat shows congested central vein,fatty degeneration and cytoplasmic vacuolation. (H& E magnification X100)

Figure 1B: Photomicrograph of liver of normal control rat shows clear central vein, well arranged hepatocytes and sinusoids. (H& E magnification X100)

Figure 1C: Photomicrograph of liver of normal rats treated with ACS(500mg/kg b.w) shows well arranged hepatocytes in between sinusoids, with clear central vein. (H& E magnification X100)

Figure 1D: Photomicrograph of liver of diabetic rat treated with ACS (500mg/kg b.w.) shows restoration of hepatocytes structure to near normal, still little congestion of central vein seen.(H& E magnification X100)

Figure 1E: Photomicrograph of liver of diabetic rat treated with Glibenclamide (5mg/kg b.w) shows restoration of hepatocytes structure, clear sinusoids and reduction in fatty degeneration. (H& E magnification X100) .

The elevated SGOT, SGPT, ALP levels in STZ induced diabetic animals were significantly reduced ($p<0.001$) in comparison with diabetic control (positive control) and Glibencl-amide treated groups (Table 3). Meanwhile non-diabetic animals (group IV) treated with ACS 500 mg/kg b.w. showed no disturbances in the levels of plasma glucose, SGOT, SGPT, ALP in comparison with negative controls.

Liver histopathology of treated animals

Examination of the stained sections of the liver of STZ induced diabetic rats revealed necrotic changes including nucleus and cytoplasmic vacuolation, hepato-cytes and sinusoids fragmentation, vascular congestion of the central vein and fatty degeneration (Fig.1.A). The negative controls (Fig.1.B) and non-diabetic ACS treated (group IV) animals (Fig.1.C) showed normal cyto-architecture of liver tissue with clearly defined hepatocytes around the central vein and well arranged sinusoids between the hepatic plate of cells. Diabetic rats treated with ACS (group V) and with Glibenclamide (group III), also showed the normal restoration of liver cyto-architecture (Fig.1 D & E) which was almost similar to control group of rats.

DISCUSSION

In our study, diabetes was induced in rats by single intraperitoneal injection of STZ at a dose of 45mg/kgb.w. and the anti-diabetic activity of *Costus spicatus* and its effect on liver histology and enzymes of liver function was studied. Diabetes mellitus is associated with progressive metabolic derangement, worsening glycemic control, and morphological changes of in the liver, pancreas and other organs (Cook et al., 2005; Cristina et al., 2008). In diebetic individuals liver enzymes such as SGOT, SGPT and ALP are present in higher concentrations in the normal hepatocytes of the liver and these enzymes are leaked into the circulation as a result of damage to cell membrane of hepatocytes (Ahsan et al., 2009).

The present study found that, ACS administration to STZ induced diabetic animals significantly reduced the abnormal fasting blood glucose level which was comparable to control. This decreased plasma glucose levels may be correlated with decreased gluconeogenic activity (Oliveira et al., 2005) which may be the reason for weight gain in ACS and Glibenclamide treated diabetic animals (Pandikumar et al., 2009). The elevated levels of SGOT, SGPT in serum are an indication of damaged liver tissue, administration of ACS improves the liver function by decreasing the levels of SGOT, SGPT in diabetic treated rats, indicating its hepatoprotective effect. ALP acts as a marker for biliary function (Santhosh Kumar et al., 2008). Reduction of ALP levels comparable with control in ACS treated diabetic animals further confirms its hepatoprotective effect.

In ACS treated non-diabetic animals the levels of hepatic enzymes were not disturbed which reveals the non-toxic nature of ACS (Godam et al., 2014). Though Glibenclamide treatment restored the normal levels of liver enzymes like ACS, but liver tissue showed the presence of vascular congestion of central vein and few hepatocyte nuclei vocalizations.

In conclusion, both ACS and Glibenclamide of STZ induced diabetic animals restored the normal plasma glucose levels and SGOT, SGPT and ALP levels. But ACS restored the normal plasma glucose and SGOT, SGPT and ALP levels without damaging the liver.

Conflicts of interest: The authors stated that no conflicts of interest.

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International Journal of
Life Sciences
International Peer Reviewed Open Access Refereed Journal

Special Issue A 11: January 2018
UGC Approved Journal No 48951

Original Article | Open Access

Molecular studies in Rheumatoid arthritis patients for determination of Cardiovascular disease (CVD) risk

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Manuscript details:

Available online on
http://www.ijlsci.in

ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

Editor: Dr. Arvind Chavhan

Cite this article as:
Pradeepa K and Lakshman Kumar B (2018) Molecular studies in Rheumatoid arthritis patients for determination of Cardiovascular disease (CVD) risk, *Int. J. of Life Sciences*, Special Issue, A11: 67-72.

ABSTRACT

Among the serious complications people with rheumatoid arthritis (RA) experience, cardiovascular disease heads the list. Having RA doubles the risk of most heart problems, including heart attack, stroke and atherosclerosis - the build-up of fat, cholesterol and cellular debris (plaque) on blood vessel walls. Hence the present study targeted the I/D polymorphism in angiotensin 1 converting enzyme (ACE) gene using Insertion specific PCR and methylation status of angiotensin 1 converting enzyme (ACE) gene using methylation specific PCR in 30 RA patients confirmed by Rheumatoid factor (RF) and erythrocyte sedimentation rate (ESR) values. ID genotype was the predominant genotype in patients and controls. II genotype was higher in controls compared to patients. Methylation pattern analysis using MS-PCR suggested equal distribution of methylated and unmethylated regions of ACE gene in patients and controls thus suggesting lack of CVD risk. But in order to confirm, a higher sample size needs to be further probed.

Keywords: Angiotensin 1 converting enzyme (ACE), rheumatoid arthritis, Cardiovascular disease (CVD), Methylation.

INTRODUCTION

Rheumatoid arthritis is a chronic systemic inflammatory disease of undetermined aetiology, involving primarily the synovial membrane and articular structures of multiple joints. The disease is often progressive and results in pain, stiffness and swelling of joints. In later stages deformity and ankylosis develop. The process involves an inflammatory response of the capsule around the joints (synovium) secondary to swelling (turgescence) of synovial cells, excess synovial fluid, and the development of fibrous tissue (pannus) in the synovium. Rheumatoid factor (RF) is an antibody that can be found in the blood of 80% of people with rheumatoid arthritis. The insertion/deletion (I/D) polymorphism in ACE gene refers to an Alu repetitive sequence of 287 bp long, in intron 16, resulting in three genotypes, DD and II homozygotes and ID heterozygotes.

A significant relation between the number of methylated CpG sites and the ACE mRNA expression in various cell lines has been previously reported (Riviere *et al*., 2011). ACE gene is discussed as a common factor for the known relation between major depression and cardio- vascular disease, but the underlying mechanisms are poorly understood. Epigenetic alterations, probably via environmental influences as early stressful life events might be one explanation for these observations. The main objectives of the present study were to find an association between RA and CVD risk through analysing the ACE I/D genotype polymorphism and methylation pattern in ACE gene in Arthritis patients and to compare the obtained results with normal healthy controls using relevant statistical tools.

MATERIALS AND METHODS

Study participants and characterization

About 30 venous blood samples were collected from clinically confirmed RA patients in EDTA coated vacutainers from 'DEEPA MICROLAB' Erode, Tamil Nadu. The patients were categorized as RA based upon Rheumatoid factor (RF) values and ESR values. Thus obtained blood samples were brought to the laboratory and stored at 4^{o}C for DNA isolation.

Genomic DNA extraction

One mL of whole blood was lysed with 3mL of chilled RBC lysis buffer, vortexed for 1 minute and centrifuged at 4000rpm for 5minutes and the red supernatant was removed. This step was repeated twice to get white to pink pellet. To this 200µL of nuclei lysis buffer and 50µL of SDS were added. Followed by the addition of 3µL of proteinase K, the mixture was incubated at 65^{o}C for 2:30 hours. After this, 175µL of 5.3M Sodium chloride was added, centrifuged at 10,000rpm for 15minutes, supernatant carefully siphoned off and transferred to a new 2mL microcentrifuge tube. To this one mL of cold 100% ethanol was added and inverted ten times to precipitate the DNA. There after the tube was centrifuged at 1500 rpm for 10minutes, supernatant was removed and the pellet was resuspended in 75% alcohol. Centrifugation at 15000 rpm was performed to remove supernatant. The pellet was air dried and resuspended in 100-150µL of TE buffer and stored at -20^{0}C. The A_{260}/A_{280} values were checked to assess DNA purity through UV/Visible spectrophotometer.

Insertion specific – PCR

The isolated DNA samples were used for the amplification of ACE gene to find the distribution of DD, ID and II genotypes distribution in patients and controls using previously reported primer pairs (Tuncer *et al*., 2006). Forward primer 5'-CTGGAGACCACTCCCATCCTT 3'; reverse primer 5'-GATGTGGCCATCACATTCGTC3'. Thermal conditions for PCR consisted of initial denaturation at 95^{o}C for 10 min followed by 35 cycles of denaturation at 92^{o}C for 40 sec, annealing 58.1^{o}C for 30 sec, extension at 72^{o}C for 40 and a final extension at 72^{o}C for 10 minutes.

Bisulphite treatment of genomic DNA:

The DNA (up to 2µg) was digested with 20µL of restriction enzyme *Hha*I prior to bisulphite treatment. Then 2µL of 3M NaOH was added and denatured the DNA for 15 min at 37^{o}C. Further to the sample was added 208µL of urea/ bisulphite solution and 12µL of 10mM hydroquinone to make up final concentration of 5.36M urea, 3.44M bisulphite and 0.5% hydroquinone and the mixture was incubated overnight at 55^{o}C. After this, the mixture was purified using ready to use columns supplied by Helini Biomolecules, India.

Qualitative methylation-specific PCR analysis of ACE gene

To analyze the status of methylation of ACE gene, primers were designed using MethPrimer software programme from reference sequence of ACE gene obtained from NCBI. Criteria used as defined by the software: Island size > 100, GC Percent> 50.0, Obs/Exp> 0.60. There was no *Hha*I restriction site in ACE gene region studied as reported by NEB cutter. Primer pairs for methylated ACE, forward methylated primer 5'TATATTTTTATTTTCGGTAGGCGAC-3'; reverse methylated primer 5'-CTAAAAACCTAACGACCTCCGA-3'. Primer pairs for unmethylated ACE, forward unmethylated primer 5'- TATTTTTATTTTTGGTAGGTGATGT-3'; reverse unmethylated primer 5'-AACCTAAAAACCTAAC AACCTCCA-3'.

Thermal conditions for PCR consisted of an initial denaturation at 94^{o}C for 10 min, 38 cycles of denaturation at 95^{o}C for 40 sec, annealing at 49^{o}C for 30 sec, extension at 72^{o}C for 40 sec and a final extension at 72^{o}C for 7 minutes.

Statistical analysis:

Statistical analysis for ESR and genotypes were performed using Vassarstats statistical software

(http://vassarstats.net/). Allele frequency was calculated using the formula: Allele frequency = [(homozygous allele*2) + heterozygous alleles] / (total*2).

RESULTS

The demographics of patients and controls with ESR values were presented in Table 1. ESR values of patients and controls were compared using student's '*t*' test which revealed a significant difference ($p<0.0001$, Table 2).

Table 1: Demographics of patients with ESR values

Sample No	Age	Gender	Esr (mm/hr)	Patient / Control
S1	55	Male	20	Patient
S2	60	Female	20	Control
S3	48	Female	62	Patient
S4	60	Female	10	Control
S5	75	Male	22	Patient
S6	29	Female	8	Control
S7	31	Male	52	Patient
S8	55	Male	12	Control
S9	62	Female	44	Patient
S10	42	Male	7	Control
S11	63	Male	28	Patient
S12	39	Female	15	Control
S13	50	Female	34	Patient
S14	29	Male	7	Control
S15	42	Male	32	Patient
S16	12	Male	10	Control
S17	44	Female	36	Patient
S18	48	Female	16	Control
S19	61	Male	28	Patient
S20	53	Male	11	Control
S21	45	Female	86	Patient
S22	60	Male	14	Control
S23	52	Male	40	Patient
S24	62	Female	18	Control
S25	55	Female	38	Patient
S26	45	Female	16	Control
S27	51	Male	48	Patient
S28	52	Male	13	Control
S29	28	Female	32	Patient
S30	55	Female	7	Control
S31	43	Male	46	Patient
S32	50	Female	14	Control
S33	59	Female	110	Patient
S34	63	Male	12	Control
S35	55	Male	50	Patient
S36	55	Male	11	Control
S37	47	Female	92	Positive
S38	56	Female	15	Control
S39	16	Male	22	Patient

Table 1 : Continued…

Sample No	Age	Gender	Esr (mm/hr)	Patient / Control
S40	17	Male	8	Control
S41	42	Female	80	Patient
S42	76	Male	9	Control
S43	63	Female	28	Patient
S44	31	Male	12	Control
S45	39	Female	26	Patient
S46	33	Female	56	Patient
S47	60	Female	52	Patient
S48	56	Female	56	Patient
S49	53	Female	48	Patient
S50	57	Female	50	Patient
S51	60	Female	9	Control
S52	47	Female	10	Control
S53	75	Male	13	Control
S54	61	Male	17	Control
S55	44	Female	22	Control
S56	32	Male	9	Control
S57	33	Female	7	Control
S58	57	Female	21	Control
S59	53	Female	20	Control
S60	63	Female	24	Control

Table 2: Student's '*t*'- test comparison of ESR values

$Mean_a$- $Mean_b$	'*t*'	df	P	One- tailed	<.0001
-34.1667	-8.54	31.24		Two- tailed	<.0001

Table 3: ACE genotypes observed in patients and controls

RA patients	II Genotype	I/D Genotype	DD genotype
	3	20	7
Controls	12	12	6
	II/ID genotype OR 0.15 95% confidence interval from 0.0351 to 0.6418 P=.016	ID/DD OR 1.4286 95% confidence interval from 0.3876 to 5.2646 P=0.840	II/DD OR 0.2143 from 0.0403 to 1.1386 p=0.142

Table 4: Methylation pattern of ACE gene in patients and controls

	Methylated	Unmethylated	Methylated+ Unmethylated
RA patients	11	10	9
Control	12	10	8

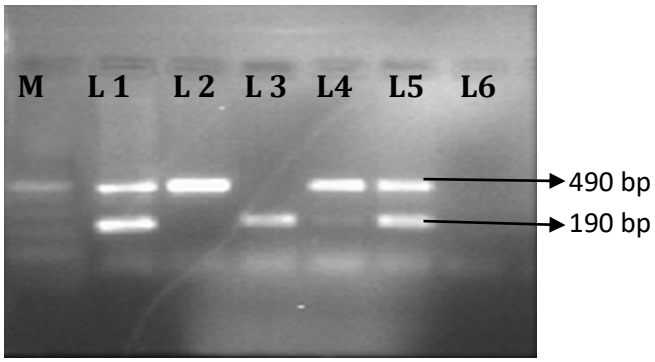

Figure 1: ACE I/D polymorphism in RA patients (positive)
M: 100 bp marker ; Lane: 1, 5- ID genotype ;
Lane: 2, 4- II genotype ; Lane: 3- DD genotype

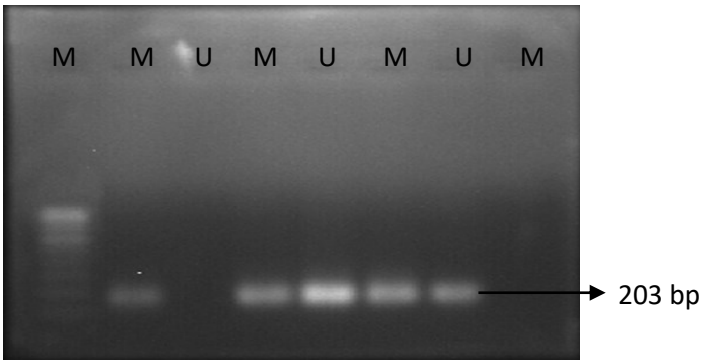

Fig 2: Methylation pattern in RA patients (positive)
Lane1 M: 100 bp Marker ; Lane 2, 4, 6: Methylated ; Lane 3, 5, 7: Unmethylated

Insertion – specific PCR

SNP analysis of ACE gene in RA patients revealed that there were 20 I/D genotypes, 3 II genotypes and 7 DD genotypes (Figure 1), whereas in controls there were 12 I/D genotypes, 12 II genotypes and 6 DD genotypes. Concerning the allele frequency both fitted in Hardy-Weinberg equilibrium with allele frequencies of 0.56 (patients) and 0.40 (controls) for D allele and 0.44 (patients) and 0.60 (controls) for I allele. The distribution of II genotype significantly differed between patients and controls ($p<0.05$, Table 3, Fig. 1).

Qualitative methylation specific PCR

ACE gene was found to be methylated in 36%, Unmethylated in 33.33% and both methylated and unmethylated in 30% of patients (Fig. 2). In controls, ACE gene was methylated in 40%, 33.33% were unmethylated and 26.66% had both methylated and Unmethylated (Table 4).

DISCUSSION

RA is a multiorgan chronic and complex disease with an autoimmune basis. Cardiovascular disease (CVD) is considered an extra-articular manifestation (EAM) (Sandoo *et al*., 2011) and a major predictor of poor prognosis of RA (Demaria 2002).

This study was undertaken to investigate cardio vascular disease risk in RA patients. Patients with RA are 30% to 60% more likely to suffer a CV event compared with the general population (Watson *et al*., 2003, Han *et al*., 2006), especially myocardial infarction (Turesson *et al*., 2004, Solomon *et al*., 2003, Wolfe *et al*., 2003). CVD accounts for 30–50% of all deaths in RA patients (Sandoo *et al*., 2011). Cardiovascular disease (CVD)-associated mortality risk is increased in both men and women with seropositive RA (Jonsson*et al*., 1999).

In the present study, 30 patient and 30 control samples were collected. Among the 30 patient samples 18 were females and 12 were males. RA is three times more frequent in women than men (Scott *et al.,* 2010). It usually strikes women between the ages of 25 to 50. Approximately 1.3 million American adults have RA, with women outnumbering men 2.5-to-1. From 1985 to 1994, the incidence of rheumatoid arthritis was 36.4 per 100,000 women, but from 1995 to 2004, that number increased by nearly half to 54 per 100,000 women. The incidence in men, however, stayed about the same, going from 28.6 to 29.5 per 100,000 over the same two decades.

ACE could be involved in the pathogenesis of cerebro-vascular disease by several biological mechanisms, including activation of angiotensin I and inactivation of bradykinin, resulting in decreased tissue perfusion, vascular smooth muscle cell growth, and stimulation of plasminogen activator inhibitor type 1. (Ehlers *et al*., 1989; Mizuno *et al*., 2003).

Distribution of both variant alleles (D and I) was similar among the patients and the controls. Similar distribution of the variant alleles led to lack of any significant association between the *ACE* ID polymorphism and CVD risk, either in homozygotes and heterozygotes or combined forms. In the present study D allele frequency was higher in patients and controls. Also ID/DD genotypes seemed to have a slight risk based on Odds ratio value. The distribution of the ACE genotypes differs between

races and it is used as a marker in population structure analyses (Barbalic*et al*. 2004).

Koreans and Japanese have reported a relatively low percentage of the DD genotype. The frequency of the D allele is 0.406 in the Koreans (Choung *et al*. 1999) and 0.33 in the Japanese population (Yoshida *et al*. 1995), contrast to Caucasians, where the D allele frequency is higher.

In methylation analysis of the current study, 11 were methylated, 10 were unmethylated and 9 were both methylated and unmethylated in RA positive samples whereas 12 were methylated, 10 were unmethylated and 8 were both methylated and unmethylated in controls. ACE promoter methylation has been found to affect serum ACE protein levels. Higher rates of ACE methylation have been found to lower ACE serum concentration and have a protective effect on the development of CVD. The differential DNA methylation patterns in the promoter region of the ACE gene seem to affect the expression of inflammatory CVD risk marker concentrations in patients with depression. Methylation pattern has been to have an influence on serum ACE protein expression in the sample and additionally on the amount of inflammatory risk markers for CVD, as ICAM-1, E-selectin and P-selectin in patients (Zill*et al*., 2012).

In conclusion, ESR proved to be a good marker for RA disease activity in patients compared to controls. ID genotype was the predominant genotype in patients and controls. II genotype was higher in controls compared to patients. Because of a more or less similar distribution of alleles, any association with cardiovascular disease risk could not be ascertained. Also methylation pattern analysis using MS-PCR suggested equal distribution of methylated and unmethylated regions in patients and controls, resulting in less significant findings. But in order to confirm the above findings, a higher sample size needs to be studied.

Conflicts of interest: The authors stated that no conflicts of interest.

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International Journal of
Life Sciences
International Peer Reviewed Open Access Refereed Journal

Special Issue A 11: January 2018
UGC Approved Journal No 48951

Original Article Open Access

Predicted side effects of NPC1 protein inhibitors

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Manuscript details:

Available online on
http://www.ijlsci.in

ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

Editor: Dr. Arvind Chavhan

Cite this article as:
Cherian Prakash V and Kuppanan Suresh (2018) Predicted side effects of NPC1 protein inhibitors, *Int. J. of. Life Sciences*, Special Issue, A11: 73-76.

ABSTRACT

Niemann-Pick C1 (NPC1) gene encodes a large protein that transports low-density lipoproteins to late endosomal / lysosomal compartments where they are hydrolyzed and released as free cholesterol. NPC1 facilitates Ebola and Marburg virus entry into the cytoplasm, serving as an intraluminal receptor after these viruses have been internalized. The recent Ebola virus outbreak clearly showed the lack of available vaccines to treat infected patients and to stop the spread necessitates the discovery of antivirals. Viral entry is the initial and an essential step in the viral replication cycle and blocking the entry into the target cell leads to suppression of viral infection and is therefore an attractive antiviral strategy. Several studies have found novel small molecule inhibitors to inhibit the interaction of Ebola virus glycoprotein (mediates viral entry into cells) with NPC1 and suggested NPC1 as antifiloviral therapeutic effect. NPC1 protein shares sequence homology with Protein patched homolog 1 and 2 which are tumor suppressor. NPC1 is being expressed in oocytes, testes and ovary at different amounts, but oocytes express far more strongly than other cell types. Therefore any therapeutic molecule developed to inhibit NPC1 may also affect the functions of other cell types where NPC1 expresses and interfere with the tumor suppressor function of PTCH 1 & 2.

Keywords: Niemann-Pick C1, Protein patched homologue 1, Ebola, lysosomal storage disease, cholesterol transport.

INTRODUCTION

Niemann-Pick C1 gene encodes a large protein involved in the transportation of cholesterol as cholesterol esters that are packaged into low-density lipoprotein (LDL) (Roth, 2006). LDL is endocytosed and transported to late endosomes and lysosomes where it is subjected to lipolysis and liberated as free cholesterols. These free cholesterols bound to an intra-lysosomal protein called NPC 2 (Naureckiene et al., 2000) to be transported to other cellular compartments. Mutation in NPC1 cause excessive accumulation of cholesterol in lysosomes within multiple tissues and induces a fatal lysosomal storage disease (Pentchev, 2004).

Besides this, recent researches have found that ebola virus glycoprotein interaction with NPC1 facilitates the Ebola virus entry into the cytoplasm. Ebola virus infection causes acute hemorrhagic fever in humans with mortality rate exceeding 50% (Leroy et al., 2009; Ray et al., 2004; Sullivan et al., 2000).

Currently, no FDA approved drug to treat ebola viral infection available in clinical setting and the treatment for Ebola infection primarily limited to palliative care and practicing methods to prevent transmission. Of the various approaches, inhibiting the entry of virus into cells is an attractive approach since entry of virus is an initial and essential step in the replication of virus. Investigations on the mode of entry of ebola virus revealed that, NPC1 is an absolute requirement for the entry into the cytoplasm (Herbert et al., 2015; Bruchez Anna 2012; Cote M et al., 2011). Several investigations explored small molecules as inhibitors of NPC1 (Cote M et al., 2011; Bruchez Anna, 2012; Basu A et al., 2015). The present study was aimed to predict the undesirable effects of inhibition of NPC1 by small molecule inhibitors through an *In silico* approach.

MATERIALS AND METHODS

Niemann Pick C1 amino acid sequence was retrieved from NCBI (GenBank: ANN44507.1). This sequence was subjected to blastp analysis leaving default parameter undisturbed. Blastp search returned a list of similar proteins including isoforms of NPC1. All the isoforms were ignored and two proteins, Protein patched homolog 1 (PTCH1) and Protein patched homolog 2 (PTCH2) were selected.

Kyoto Encyclopaedia of Genes and Genomes (KEGG) database was used to find a signalling pathway that is associated with NPC1 and PTCH1 and PTCH2. Amazonia! database was used to retrieve information pertaining to expression of NPC1 in different cell types.

RESULTS AND DISCUSSION

According to KEGG, NPC1 protein was associated with three pathways which include fat absorption and digestion, lysosome-phagocytosis and antidyslipidemic agents. In fat absorption and digestion pathway, NPC1 seemed to assist in transport of intestinal and hepatic cholesterol transport. If this protein is inhibited by small molecule inhibitors, could lead to poor cholesterol absorption in intestine and liver. However, recent research disproves this notion and suggests the existence of an alternate pathway for cholesterol uptake in humans (Dixit et al., 2007). Mutation in either NPC1 or NPC2 results in a disease called Niemann pick disease in which endocytosed cholesterol becomes sequestered in late endosomes / lysosomes and this consequently causes progressive neurodegenration in mice (Yu et al., 2005; Ong et al., 2001). The NPC1 is a polytopic protein of late lysosome / endosome (LE/L) limiting membrane whereas NPC2 is a soluble protein in the LE/L lumen.

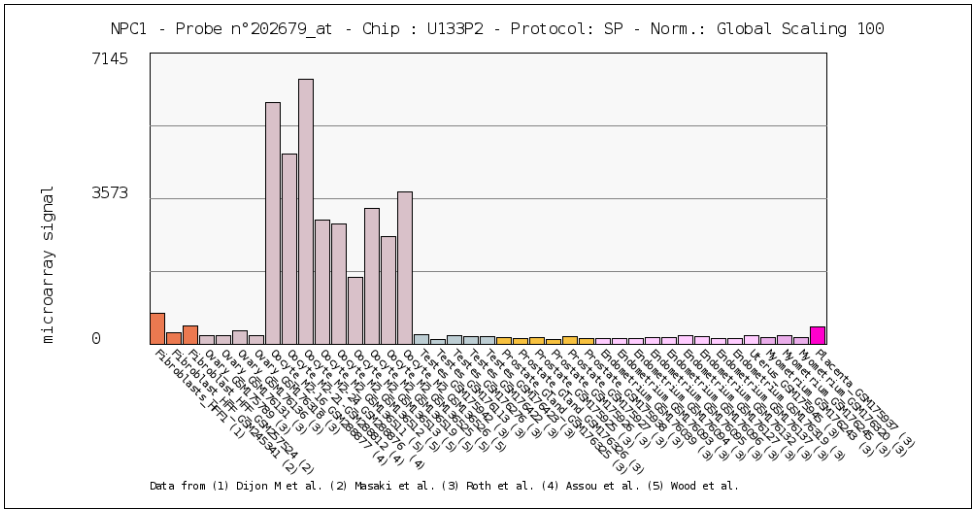

Figure 1: NPC1 expression in oocytes as shown by Amazonia!

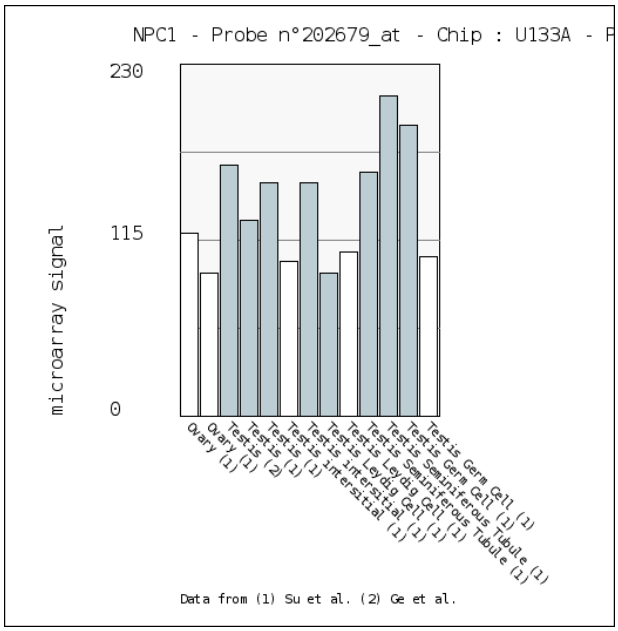

Figure 2: NPC1 expression testis as shown by Amazonia!

Both NPC1 and NPC2 act in tandem and help the transport of cholesterol from LE/Ls. Hence a defect in either NPC1 or NPC2 results in accumulation of cholesterol in LE/Ls, therefore there exists a risk that inhibition of NPC1 by small molecule inhibitors could result in Niemann Pick C disease. In the antidyslipidemic pathways, NPC1 protein acts as triglyceride and LDL cholesterol reducing agent. Inhibition of NPC1 protein may result in accumulation of LDL cholesterol which may increase the risk of getting cardio vascular diseases. Protein patched homologue 1 and 2 are tumor suppressor proteins and according to blastn search, PTCH 1 and 2 shared sequence homology with NPC1. Greer et al. (1999) reported a significant sequence homology throughout NPC1 transmembrane spans with transmembrane domain of plasma membrane PTCH1. Therefore, small molecule inhibitors inhibiting NPC1 may also inhibit PTCH1 which is a tumor suppressor gene.

Amazonia! search with NPC1 revealed that, NPC1 gene expresses strongly in oocytes (Fig. 1) (Masaki et al., 2007; Assou et al., 2009; Wood et al., 2007). Inhibitors which inhibit NPC1 may also affect the maturation of oocytes thereby affecting reproductive health. In addition to this, testis also expresses NPC1 (Fig. 2) (Ge et al., 2005; Su et al., 2004). The NPC1 knock out studies in mice revealed that, NPC1 $1^{-/-}$ male mice infertile because of partial arrest of spermatogenesis and spermatozoa also showed morphological head abnormalities and are unable to fertilize an oocytes *in vitro* (Fan et al., 2006)..

CONCLUSION

Our study concludes that, while exploring inhibitors to inhibit NPC1, it is recommended to check for embryonic and oocytes toxicity using embryonic and oocytes cell lines and animal models. We feel that, it is also necessary to carry out extensive studies on animal models to elucidate the effect of NPC1 inhibitors have on cancer. Further, all volunteers for clinical trials must be tested for Niemann' pick type C disease.

Conflicts of interest: The authors stated that no conflicts of interest.

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International Journal of
Life Sciences
International Peer Reviewed Open Access Refereed Journal

Special Issue A 11: January 2018
UGC Approved Journal No 48951

Original Article Open Access

Clinical implication of ACE gene polymorphism in Systemic Lupus Erythematosus patients

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Manuscript details:

Available online on
http://www.ijlsci.in

ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

Editor: Dr. Arvind Chavhan

Cite this article as:
Chandirasekar R, Uthayakumar V, Anitha E, Mekala S, Mathivanan R, Raghadevi M (2018) Clinical implication of ACE gene polymorphism in Systemic Lupus Erythematosus patients , *Int. J. of. Life Sciences*, Special Issue, A11: 77-84.

ABSTRACT

Systemic Lupus Erythematosus is a complex genetic disease, associated with environmental and genetic factors. Angiotensin-converting enzyme (ACE) plays an important role in the development of SLE, is expressed in a wide range of tissues including lung, vascular endothelium, kidney, cardiac muscles and testis. ACE gene I/D polymorphisms, particularly the DD genotype has been associated with lupus susceptibility and with risk of renal and cardiovascular disease. This study analysed the ACE gene polymorphic variants among the recruited 48 SLE patients (SLE was confirmed by Anti nuclear antibody (ANA) and dsDNA tests) associated with the cardiovascular and renal disease and 48 controls. The present study also analyzed the biochemical parameters (total serum cholesterol HDL, LDL, VLDL, TGL) and C-reactive protein (CRP) in SLE patients and controls. Our study found that, SLE patients had higher levels of cholesterol (40%) than controls and the and CRP was found to be higher in SLE patients in comparison with controls and established normal reference range (<5mg/L). The distribution of genotype in SLE patients was DD 62.50%, ID 20.83% and II 16.66%. A significantly higher frequency of DD genotype (p<0.0004) was observed in SLE patients in comparison with controls.

Keywords: Systemic Lupus Erythematosus, ACE, CRP, Anti nuclear antibody, Cholesterol.

INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a complex, chronic, multi-system autoimmune disease which varies in prevalence and incidence depending on ethnicity (Lau *et al.,* 2006; D'Cruz *et al.,* 2007). While the precise etiology of SLE still remains vague, genetic predisposition, environmental and hormonal factors are deemed to play important roles in its pathogenesis. Severity, acquisition risk and clinical manifestations of this disease can vary by ethnicity, geography and sex, with a prevalence

that is higher in women during their childbearing ages and some non-European populations such as African Americans, Hispanics and Asians (Lau *et al.,* 2006; Voskuhl, 2011). Genetic, hormonal, environmental and immunoregulatory factors contribute to the expression of the disease (Kammer *et al.,* 1999).

Genetic studies have demonstrated a strong contribution of many gene variants to SLE incidence and the clinical manifestations of SLE (Wong and Tsao, 2006; Hom *et al.,* 2008; Flesher *et al.,* 2010).

SLE is a complex prototypic autoimmune disease that predominantly affects women; the hallmark of SLE is the generation of auto antibodies that react with self nuclear and cytoplasmic antigens, culminating in immunologic attacks to body organs (Petri, 2006). With very distinct forms of presentation, includes articular and mucocutaneous involvement, renal disease, hematological abnormalities and central nervous systems disease (Cervera *et al.,* 1993). It is a multisystem disease with a variable course and a wide range of clinical manifestations (Gudmundsson and Steinsson, 1984). Among a range of factors that are thought to be contributing to the pathophysiology of SLE, chronic inflammation is thought to play a pivotal role in the pathogenesis of SLE (Alarcon-Segovia *et al*., 2005).

Several studies have shown that the C-reactive protein (CRP) levels in SLE patents are abnormally elevated both in the absence and presence of infection (Royand Tan, 2001). The value of using CRP to monitor SLE disease activity has remained in practice. Serum lipids can be used to identify patients with SLE who are at increased risk of renal dysfunction (Font *et al.,* 2001).

Rheumatoid factor (RF) and anti nuclear antibody (ANA) are associated with cardio vascular disease (CVD) events and overall mortality both in those with and without rheumatic diseases. Inflammation (as studied through elevations in CRP and/or Erythrocyte Sedimentation Rate (ESR)) contributes to increased risk of CVD, both in patients with autoimmune diseases (Danesh *et al*., 2004). ESR develops in 5–20 % of patients with SLE and renal involvement (Cortes-Hernandez *et al.,* 2005).

Patients with SLE also develop arterial stiffness, but it is uncertain if arterial stiffness occurs independently of age, hypertension, renal function, or atherosclerosis (Yildiz *et al*., 2008). Renal injury in SLE is one of the most serious complications and its pathogenesis has not yet been completely clarified (Sprovieri and Sens, 2005). Angiotensin-converting enzyme (ACE) plays an important role in the development of SLE, expressed in a wide range of tissues including lung, vascular endothelium, kidney, cardiovascular and testis (Kaufman *et al*., 2001). ACE catalyses the conversion of Angiotensin I to Angiotensin II by its metalloproteinase enzymatic activity and plays a major role in the renin–angiotensin and kallikrein–kininogen systems. A major component of the renin-angiotensin system, ACE is up regulated in pressure overload–induced cardiac hypertrophy as well as heart failure (Schunkert *et al*., 1993). It is long recognized that autoimmune diseases such as SLE are associated with increased mortality and an increased risk of CVD, which is not explained by traditional CVD risk factors alone (Doria *et al.,* 2005).

Manzi *et al.* (1997) established in the Pittsburgh cohort that certain risk factors were more common in SLE patients with cardiovascular events than in those without events, including older age at diagnosis, longer disease duration, hypercholesterolemia, postmenopausal status (Manzi *et al*., 1999).

ACE gene I/D polymorphisms, particularly DD genotype has been associated with lupus susceptibility and with risk of renal disease and cardiovascular disease (Kennon *et al*., 1999). Inhibition of ACE induces regression of cardiac hypertrophy independent of load (Linz *et al*. 1992) and prevents dilation and remodelling of the ventricle after myocardial infarction (Pfeffer *et al*., 1992). Inflammatory mediators and an array of inflammatory cells can induce a broad spectrum of clinical manifestation (Kyttaris *et al*., 2005).

The purpose of this study was to determine the relationship between the biochemical profile, autoantibodies, and ACE gene polymorphisms in relation to renal and cardiac disease.

MATERIALS AND METHODS

Sample selection

The study group comprised of 48 SLE female patients and equivalent number of healthy normal control subjects, matched for age (±5), gender, and socio-economic conditions. The samples were collected from SLE patients belonged to Coimbatore, Madurai, Erode districts in Tamil Nadu, South India. The work was

followed and carried out in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. After obtaining written informed consent from all participating subjects, information regarding health status, habit and occupation was recorded. Individuals who reported a history of chronic or acute diseases were excluded from the study. About 4 ml of peripheral blood sample was collected from SLE and control subjects in heparinzed and EDTA coated vaccutainers. The blood samples were brought to the laboratory within 24 hours for analysis of immunological, biochemical and genotypic analysis.

The immunological and biochemical parameters (Serum Cholesterol, ANA, RF, ESR and CRP) were analyzed through a commercial laboratory (Religare SRL Ranbaxy Mumbai India) and X-rays (for heart and lungs) and ECG (for the heart, pulmonary functions for the lungs) were done from Sugapriya Hospital Madurai, India.

Genotyping of ACE I/D Polymorphism

Genomic DNA was isolated according to the standard protocol (Miller *et al*., 1988). The specific segment of ACE gene was amplified by using the previously reported primers (Salem and Batzer, 2009). The primer sequences were as follows, Sense primer: 5'- CTGGAGACCACTCCCATCCTTTCT -3' and antisense primer: 5'-GATGTGGCCATCACATTCGTCAGAT-3'. PCR reaction was performed in a final volume of 25 µl with 0.25µl of forward and 0.25µl of reverse primers, followed by 200ng of template DNA, 1.25units of Taq DNA polymerase, 2mM dNTPs in 2mM $MgCl_2$. Thermal conditions consisted of an initial denaturation at 94^{o}C for 5 minutes and 35 cycles of denaturation at 94^{o}C for 1 minute, annealing at 58^{o}C for 30 seconds, extension at 72^{o}C for 1 minute and final extension at 72^{o}C for 8 minutes. A 190bp amplicon was observed in case of homozygous DD genotype, 490bp in case of homozygous II genotype and heterozygous DI genotype.

All samples that were identified initially as a DD genotype were reanalyzed using an insertion-specific primer pair, as reported by Lindpaintner *et al.* (1995). Oligonucleotide sequences specific for I alleles primers were 5'-TGGGACCACAGCGCCCGCCACTAC-3' and 5'-TCGCCAGCCCTCCCATG CCCATAA-3'. PCR was performed with an initial denaturation at 94^{o}C for 1 minute and for 30 cycles of denaturation at 92ºC for 40 seconds, annealing at 63ºC for 40 seconds, extension at 72ºC for 40 seconds and a final extension at 72ºC for 5 minutes. The PCR products were separated by 8% polyacrylamide gel in 1X TBE buffer at 65V/cm for 1:45 hours. A 335 bp band was obtained only in the presence of the I allele and no bands were detected for samples with DD genotype

Statistical Analyses

Statistical analysis was performed with SPSS 16.0 for windows. The distribution of genotypes in all groups was tested for deviation from Hardy–Weinberg equilibrium. The Odds ratio (OR) and 95% Confidence Intervals (95% CI) were calculated with statistical significance at p-value <0.05 (J Martin Bland and Douglas G Altman Statistics Notes: The odds ratio BMJ 2000; 320:1468). Chi-square analysis ($\chi 2$ tests) was used to test the association between the polymorphic variants and biochemical parameters among the controls and experimentals. $p < 0.05$ was used as the criterion of significance. The association between clinical manifestation, production of autoantibodies and polymorphism distribution in patients with SLE was determined by Fisher exact test.

RESULTS AND DISCUSSION

The mean age of SLE patients and controls was 45.11 ± 11.50 and 44.18±11.50 respectively. In this study 40% of the SLE patients had increased total cholesterol levels and were classified into two groups based on cholesterol level such as SLE high (>200 mg/dL) and SLE low (<200 mg/dL) (Table 1). In Patients with high and low cholesterol levels, high TGL levels showed corresponding high levels of LDL and VLDL suggesting a correlation between these levels but HDL levels in both high and low level patients remained unaffected by total cholesterol level.

RF and ESR level were analysed in the SLE and control subjects and found that, in SLE subjects ESR 53.45±10.32 and RF 119±199.19 were significantly (P<0.05) increased as compared to control subjects (Table 1 and 2). CRP levels as expected was higher in all the SLE patients than the normal reference range (<5 mg/L) (Table 2). ANA score was 4+ in severe SLE patients and 1+ in low severity patients (Table 2). The patients in SLE high and SLE low groups had insignificant difference in the levels of CRP and ESR, but had high and low levels of RF in 4+ and 1+ patient respectively which points towards a positive correlation of ANA and RF.

The antinuclear antibody test is positive in almost all individuals with SLE (97%) and in the present study it was found to be elevated in all the SLE patients as compared to controls (Table 2).

Chi square analysis revealed that there was significant difference in the distribution of DD genotype between controls and SLE patients (Table 3). The patients with DD genotype had high total cholesterol levels suggesting a relationship between the two. Gel electrophoresis of amplified PCR products showed 490 bp and 190 bp products, corresponding to the PCR amplification of I and D alleles, respectively (Figure 2). The distribution of ACE genotypes in the study population was as follows 62% DD, 20.83% ID and 16.66% II. The frequency of allele D was 0.72 and I allele was 0.27.

Table 1: Immunological and biochemical profile in control and SLE subjects

Particulars	Age	ESR (mm at I hr)	HDL 35-60 mg/dL	LDL <100 mg/dL	VLDL 7.0-35 mg/dL	TGL 30-200 mg/dL	Total Cholesterol 30-200 mg/dL	CRP<5 mg/dL
Controls	44.18 ±11.50	15.95 ±5.55	44.23 ±11.00	85.10 ±18.57	24.73 ±7.75b	135.75 ±50.49	146.90 ±40.45	6.039 ±5.55
SLE High	46.77 ±11.25	53.45 ±10.32	41.04 ±16.50	129.49 ±28.14[bc]	40.67 ±22.55[abc]	184.09 ±83.35[abc]	233.95 ±33.99[abc]	10.66 ±7.24[bc]
SLE Low	43.46 ±12.58	62.38 ±42[abc]	43.57 ±27.12	88.95 ±24.10	24.13 ±6.08	127.31 ±33.48	151.15 ±129.02	6.59 ±4.40

[a] Homogeneity Variances are significant compared to the controls and low level of cholesterol in the experimental subjects (ANOVA)

[b] Bonferroni test were used an the value are compare to the controls and low level of the experimental subjects

[c] post hoc analysis (Dunnett analysis) were used and values are significant (p<0.05) to the one way ANOVA.

Table 2: Immunological and biochemical profiles in control and SLE subjects

Particulars	RF (IU/ml) <40	ANA,AB-IFA,HEP2	Anti CCP antibodies (U/ml) <15.0	DsDNA Test IU/ml 0.0-4.2	CRP
Controls	34.52 ±20.62	N	14.65 ±9.4	2.37±2.12	5.26 ±3.98
SLE High	119.35 ±199.19	4+	32.48 ±63.73	46.29±25.44	21.78 ±2097
SLE Low	191.21 ±234	2+	26.98 ±51.89	15.25±12.15	45.52 ±47.78

Table 3: ACE Gene polymorphisms identified in controls and SLE subjects

Subjects	**No**	**Genotypes**			***P*<0.05**	**I allele n (%)**	**D allele n (%)**	**P(χ2)**	**OR (95%CI)**
		II n (%)	ID n (%)	DD n (%)					
SLE patients	48	8(16.66)	10(20.83)	30(62.50)	χ2=15.56 (0.0004)	26(27.08)	70(72.91)	13.55 (0.0002)	3.05 (1.67-5.57) P<0.0001
Controls	48	14(29.16)	23(47.91)	11(22.91)		51(53.12)	45(46.87)		
SLE and controls Genotypic association and the risk ratio	II/DD RR 1.79 (1.19-2.87) **Odds ratio** 4.77 (1.57-14.48) (χ2)P<0.004 (F test)P<0.005		II/ID RR 0.89(0.55-1.44) **Odds ratio** 0.76 (0.24-2.38) Pearson P=0.63 Log odds -0.27	ID/DD RR 2.31(1.38-3.89) **Odds ratio** 6.27(2.27-17.29) (χ2) (F test, Pearson test)*P*<0.0002	*P*<0.0001 (F test and Pearson)	Risk ratio 1.55(1.21-1.98) Odds ratio 3.05 (1.67-5.57) χ2 *p*<0.0004, Pearson *p*<0.0002 F test *p*<0.0001			

Genotype and allele frequencies were compared with controls and Experimental subjects via χ2 test

Chi-square (χ2) is calculated and statistically significance compare the controls *p*<0.0001.

Statistically significance level <0.0001, OR (CI) = Odd Ratio and Confidential Interval.

Subjects with DD (OR) are at greater risk for SLE when compared to ID and II genotypes. The ID genotype was most prevalent genotype in control.

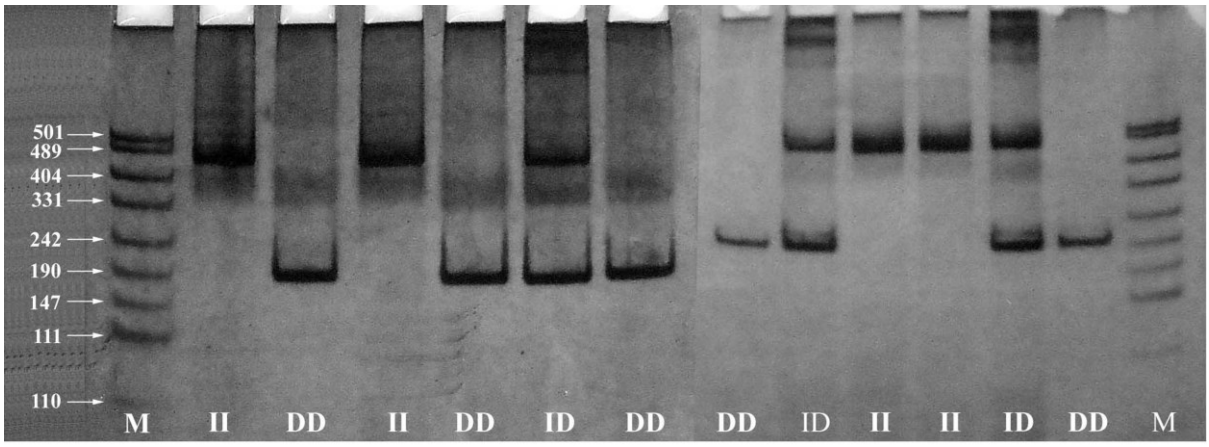

Figure 1: Homozygous DD, Homozygous II and Hetrozygous ID genotypes

From left to right A: Lane 1 PUC 19 Marker. Lane 2-II, Lane 3-DD, Lane 4-II, Lane 5-DD, Lane 6-ID, Lane 7-DD genotypes.
B: From right o left - Lane 1 PUC 19 Markers. Lane 2-DD, Lane 3-ID, Lane 4-II, Lane 5-II, Lane 6-ID, Lane 7-DD genotypes

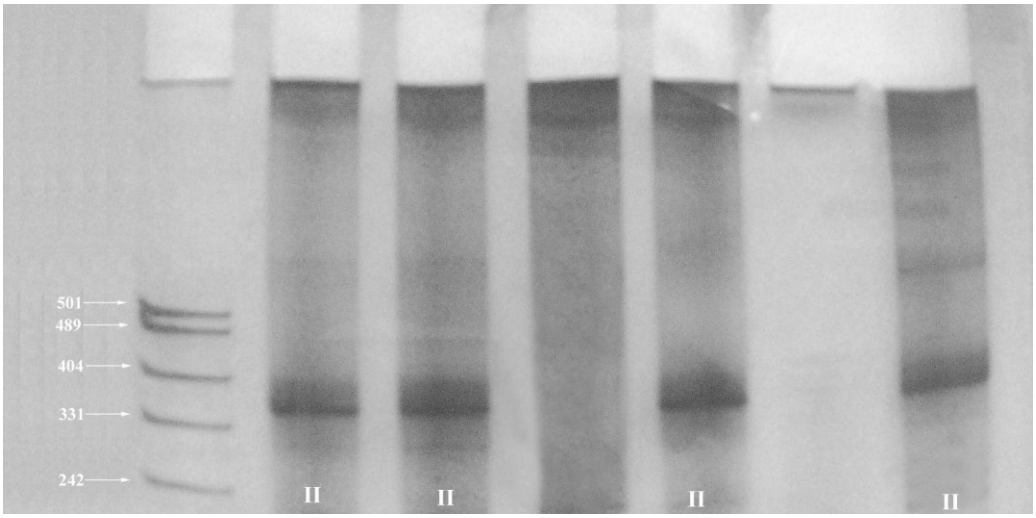

Figure 2: Determination of ACE II genotype by PCR using insertion specific ACE primers

In our study, high cholesterol levels observed in SLE patients were accompanied by an increase in CRP and ESR levels. These results are consistent with a study by Font et *al*. (2001) where SLE patients had higher total cholesterol levels which was associated with renal deterioration. RF is an antibody that is not usually present in normal individual. Out study found that, SLE patients had RF present in their body which is in total agreement with a previous study (Steiner and Smolen, 2002) which reported presence of RF in SLE disease condition. SLE is often accompanied by a number of biochemical, hematological and immunological abnormalities, though several of these abnormalities are non-specific (Dubois *et al.,* 1987).

SLE is associated with high prevalence of dyslipidemia, endothelial dysfunction, hypertension and vascular stiffness (Avalos *et al.,* 2007). The true prevalence of vascular disease in women with SLE is unknown, but could certainly be higher than that defined by cardiovascular events alone (Manzi *et al*., 1997). With improved corticosteroid and immunosuppressive SLE therapy, there is a growing pool of women at increased risk of developing cardiovascular disease, which is now one of the leading causes of death. In a large series, Gladman and Urowitz reported a 9% incidence of *angina* pectoris and/or myocardial infarction that occurred on average 89 months after the onset of SLE (Gladmann and Urowitz, 1987).

Jonsson *et al.* (1989) reported that patients with SLE who developed myocardial infarction had significantly longer duration of the disease. Previous data has suggested that ESR levels in SLE patients can be used to asses disease activity as ESR is an inexpensive diagnostic tool (Luis *et al*., 2005). SLE is an immune

complex mediated disease, which is more common in women. LDL (especially in its oxidized form) may be an important mediator of nephrosclerosis. A high triglyceride level in women and a low HDL cholesterol level in men predicted the decline of renal function (Ettinger *et al.,* 1987).

Font *et al.* (2001) showed link between hyper cholesterolemia with renal failure in a smaller cohort of patients with SLE. In our study, SLE patients had significantly raised triglyceride and LDL cholesterol levels, and significantly lower HDL cholesterol levels in comparison with controls. As women comprise as much as 90% of most SLE cohorts, it would be interesting to investigate whether the triglyceride level is also predictive of renal deterioration in patients with lupus.
The present study found DD genotype overrepresented in SLE patients than controls. In a previous study, the D allele was found to be significantly higher in the SLE patients versus non-related controls (Mattei *et al*., 1989). In another study DD allele frequency was higher in the SLE patients compared to the controls (Kaufman *et al*., 2001). Angiotensin-converting enzyme is an attractive candidate to play a role in the development of vascular pathological states. Evidence suggests that the presence of the DD genotype increases susceptibility for coronary heart disease, myocardial infarction and both diabetic and non-diabetic renal disease (Kennon *et al*., 1999). Recently two studies have identified an association with the insertion /deletion in the ACE gene with systemic lupus Erythematosus (Pullmann *et al.,* 2002). In addition to several of the variables used in this study, previously reported prognosticators of poor renal function in patients with SLE include the presence of antibodies. Renal function can be evaluated effectively in patients with SLE, by measuring serum total cholesterol levels patients with SLE, who commonly suffer from both kidney dysfunctions.

CONCLUSION

The abnormal elevation pattern of CRP in SLE patients provided the first clinical clue that variation in the CRP may contribute to the pathogenesis of SLE. The study again reemphasizes that cholesterol levels may play a role in SLE patients and may increase risk of cardiovascular and renal diseases. Also DD genotype of ACE may increase the risk of renal and CVD in such patients. Altogether taking into account an array of investigations, SLE patients may be more susceptible to CVD and renal disease risk with ACE polymorphisms having a significant role.

Conflicts of interest: The authors stated that no conflicts of interest.

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International Journal of
Life Sciences
International Peer Reviewed Open Access Refereed Journal

Special Issue A 11: January 2018
UGC Approved Journal No 48951

Original Article Open Access

Chromosomal alterations in circulated blood lymphocytes of personnel exposed with Carbon di-sulfide (CS_2)

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Manuscript details:

Available online on
http://www.ijlsci.in

ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

Editor: Dr. Arvind Chavhan

Cite this article as:
Manikantan P, Yehwanthini R, Harishankar K, Sowndharya P, Shanmugasundaram S, Sivapriyadharshini S (2018) Chromosomal alterations in circulated blood lymphocytes of personnel exposed with Carbon di-sulfide (CS_2), *Int. J. of. Life Sciences*, Special Issue, A11: 85-90.

ABSTRACT

Circulated blood lymphocytes (PBL) of 41 workers occupationally exposed to Carbon di-sulfide (CS_2) from viscose industry were investigated to find a possible relationship between CS_2 and genotoxic effects. The subjects were divided into three groups: group I consisted of 41 subjects occupationally exposed to CS_2, another 41subjects who were nearby residents of viscose industry indirectly exposed to CS_2. About 41 subjects who were directly or indirectly exposed to viscose served as controls. Ambient air concentrations of CS_2 were measured in different work places. Measures of genotoxicity including the frequencies of chromosomal aberrations (CA) and sister chromatid exchanges were analyzed in group I, II and III subjects. The subjects occupationally exposed to viscose (group I) showed higher chromosome aberrations and sister chromatid exchanges, whereas in group II CA were slightly lower in comparison with group I which may be attributed to a different rate of elimination of damaged lymphocytes as a consequence of CS_2 induced apoptotic activity. In conclusion, the results demonstrate that exposure to CS2 induces apoptosis and CA, indicating an excess cancer risk among subjects occupationally exposed to CS_2. The results also emphasize the importance of the measurement of pollutants in the work place in order to avoid genotoxic effects to the workers.

Keywords: Carbon di-sulfide, sister chromatid exchange, chromosome aberration, occupational exposure, genotoxocity

INTRODUCTION

Carbon di-sulfide (CS_2) is naturally occurring chemical substances in the environment and also an important endogenous substance in the human body. CS_2 also an industrial toxicant and solvent mainly used in the viscose, rubber and chemical manufacturing industries (Fielder and Shillaker 1981). The largest use of CS_2 is in the viscose industry where it

is used to yield from alkali cellulose. Exposure to high CS_2 concentrations in these industries may cause severe effect to human body (Ruijten et al., 1990, 1993). Chronic exposure to CS_2 can cause eye, ear, cardiovascular, nervous and reproductive system disturbances (Vanhoorne et al., 1994; Huang, 2002; Sulsky et al., 2002; Tsai et al., 2002; Tan et al., 2004; Sills et al., 2005; Nishiwaki et al., 2004; Korinth et al., 2003).

Currently, exposure level of CS_2 is mostly below 31 mg/m^3, according to Guidotti et al (1999), Hoffman (1990) and Keil et al., (1996) short time exposure also cause deceleration of intra-cardiac impulse conduction and modified arrhythmia in the coronary occlusion**.** An extensive literature is available on the toxic effects and genetic and chromosomal damage of CS_2 on circulating blood lymphocytes and human sperm samples (Liss and Finkelstein, 1996; Swaen et al., 1994; Tang and Xuan, 2003). In addition Bao et al., (1996) reported an increased incidence of chromosomal aberrations in the pronuclei of zygotes in adult female mice exposed to 10/100 mg/m^3 of CS_2 for 3 weeks and mated with unexposed male mice. *Invitro* exposure of human sperm to CS_2 concentrations of 10 µmol/L resulted in increased occurrences of chromosomal aberrations (Le and Fu 1996). Furthermore the mutagenicity and genotoxicity potential of CS_2 has been evaluated *in vitro* and *in-vivo* experiments with crucial confounding factors (Wang et al., 1999; Wabg et al., 2000; Tang and Xuan, 2003; Manikantan et al., 2010). Previously CS_2 does not exhibit mutagenic activity in bacteria with or without the presence of activation system (Beauchamp, 1983). Additional *in vitro* tests, including host mediated assay, unscheduled DNA synthesis in human fibroblasts and primary cultures of human leucocytes, are unconvincing. However, the significance of these tests cannot be properly evaluated because of methodological problems including the lack of proper positive controls (Izmerov, 1983; Struwe, 1984).

Today, chromosomal alterations in human peripheral lymphocytes (PBL) are recognized as a valuable biomarker of effect of toxic chemicals, probably the only one which has been standardized and validated (Albertini et al., 2000) method to detect the genetic level damage.

Cytogenetic studies in occupationally exposed viscose industry workers resulted in genetic damage (Medeiros et al., 2003; Manikantan et al., 2010). Early identification of hazards is crucial to reduce the exposure and carcinogenic risk. A literature survey has revealed that no investigation has been conducted on this region of workers with different cytogenetic tools based on the different levels of CS_2 exposure.

The focal aim of present study was to identify genetic alterations of viscose factory workers occupationally exposed to CS_2, Tamil Nadu, South India by analyzing the structural chromosomal aberrations (CA).

MATERIALS AND METHODS

Selection of subjects

A total of 123 subjects from various viscose industries in Tamilnadu state especially Tiruppur and Erode districts were recruited. Subjects were divided into three groups, group I consisted of 41 subjects occupationally exposed to CS_2 in viscose industry, group II comprising 41 subjects occupationally not exposed to CS_2 but they are inhabitants near viscose industries, group III consisted of 41 subjects served as controls. In addition, subjects of group I further divided based on their duration of exposure (0–3; 4–6; 7–9; 10–12; 13–15 years) to CS_2. Group I subjects were exposed to viscose minimum 8 hrs per day. Group II subjects indirectly exposed to viscose because of their two decades of residence in and around viscose industries were further sub grouped based on the number of years of residence (20–30; 31–40; 41–50; 51–60 and 61–70 years). Control subjects were normal and healthy individuals who have not been exposed with any kind of chemicals and radiation hazards. Control subjects were matched to the age of exposed groups.

CS_2 concentrations in ambient air were measured at fixed locations in the industries, and the concentrations were in the range of 0.13–1.20 mg corresponding to an 8-h time-weighted average exposure (TWA-8) of 0.9mg/m^3, which exceeds the limits of both the short-term exposure (0.6mg/m3) and TWA-8 (0.6mg/m^3). All subjects complained about acute toxic effects (eye irritation) and the presence of CS_2 was perceivable in each industry.

Analysis of Chromosome aberrations

Whole blood samples were processed to study chromosome aberrations. In brief: 0.8 ml heparinized blood were cultured in duplicate at 37^{o}C, in 5% CO_2 atmosphere, in 10 ml RPMI-1640 supplemented with 20% fetal calf serum and 0.5% PHA without antibiotics.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 3.02 software. Differences between the study groups and the control group were tested with Student's t-test, $p < 0.05$ was considered as statistically significant.

RESULTS

The demographics pertaining to different groups of exposed and controls subjects are presented in Table 1. The mean age of occupationally exposed subjects was 31.05±2.14 (group I) whereas 42 ± 2.47 for the group II (residents in and around viscose industry). The average duration of occupational exposure to viscose range from 21.8 years (group I) and 17.7 years (group II).

The amount of CS_2 in the premises of industry was found to be higher than the amount found in and around the industry (Table 2). The workers occupationally exposed to viscose had higher concentrations of CS_2 in their urine than nearby residents and controls (Table 2). Further, CA analysis revealed that, the mean amount of aberrations was higher in occupationally exposed (group I) followed by nearby residents (group II) in comparison with controls (group III) (Table 3). It was

Table 1: Demographics of exposed (Occupationally and as nearby residents)

Particulars		No of Samples	Percentage (%)
Group I (Work Duration in Years)	0-3	8	19.5
	4-6	13	31.70
	7-9	11	26.82
	10-12	7	17.07
	13-15	2	4.87
Group II (Year of Residence)	20-30	11	26.82
	31-40	13	31.70
	41-50	6	14.63
	51-60	5	12.19
	61-70	6	14.63
Controls		41	100
Gender	Male	26	63.41
	Female	15	36.58
Age (mean±SD)	Group I	31.05 ±2.14	
	Group II	42 ± 2.47	

Table 2: Comparative analysis of chromosome aberrations and SCE based on the CS_2 level in workplace environment and urine samples

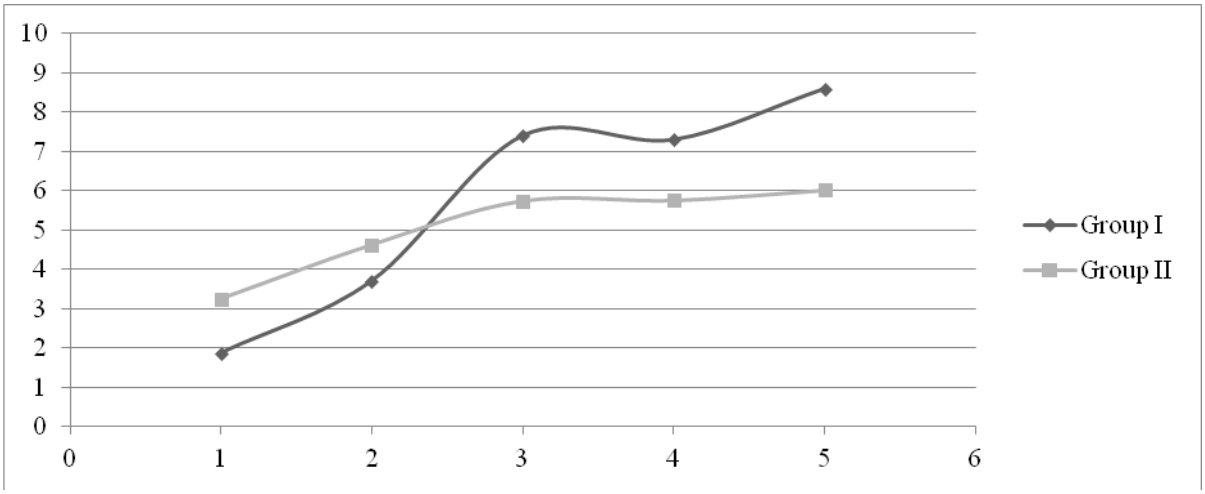
Groups	No. of Subjects	CS_2 amount in workplace mean±SD	CS2 content in urine mean±SD	Total CA (mean±SD)
I	41	1.42±0.004	1.82±0.84	5.41±2.48
II	41	0.054±0.002	1.01±0.46	4.08±3.16
III	41	0.021±0.004	0.54±0.39	1.41±2.04

Table 3: Chromosome aberration frequency and SCE in Group I subjects based on their duration of work

Particulars	0-3	4-6	7-9	10-12	13-15
Age	34.42±5.64	35.41±3.72	38.22±4.82	41.16±3.74	38.3±6.57
Work Duration	3.18±0.49	5.46±1.02	8.67±0.09	11.36±0.28	14.56±8.46
CTAs	1.46±0.87	2.57±1.03	3.67±0.05	5.62±1.18	6.08±1.40
CSAs	0.43±0.68	1.12±0.9	4.42±1.03	9.41±1.12	7.93±0.76
Total	1.86±0.62	3.70±0.84	7.40±1.14	7.32±0.81	8.62±2.36

Table 4: Chromosome aberration frequency and SCE in Group II subjects based on their years of residence

Group II	20-30	31-40	41-50	51-60	61-70
Age	36.46±3.28	41.1±2.86	38.1±1.47	39.62±4.68	38.21±6.16
Year of Residence	23.74±1.82	33.80±2.62	46.28±2.30	52.63±3.47	63.62±4.62
CTAs	3.51±1.68	3.01±1.46	3.87±1.02	6.28±0.56	6.02±0.83
CSAs	1.02±0.48	0.96±1.04	1.87±1.06	0.89±1.01	2.46±0.96
Total	3.24±0.92	4.62±1.53	5.74±1.67	5.75±1.07	6.01±2.10



X Axis Value	1	2	3	4	5
Group I	0-3	4-6	7-9	10-12	13-15
Group II	20-30	31-40	41-50	51-60	61-70

Figure 1: Graphical representation of CA value in Group I and Group II subjects

also observed that, with an increase of age and years of exposure the chromosome aberration and the mean frequency of sister chromatid exchange increases in group I subjects (Table 3).

A similar trend was also observed with group II subjects, where the chromosome aberrations and sister chromatid exchange increases with a corresponding increase in age and years of residence in group II subjects (Table 4, Fig. 1).

DISCUSSION

The results of the present study revealed cytogenetic alterations among viscose factory workers occupationally exposed to CS_2. Our study also observed considerable amount of CS_2 always present in the work place and the presence of CS_2 in the air was always observable by olfactory perception. Safety measures and devices were introduced in the last few years in these industries, and the employees are using these protective devices during their work. But none of the workers have worn masks or similar personal safety devices equipped with specific filters for CS_2. Therefore, to assess the excess risk among these workers in the second group, we have considered the effects of low dose exposure to organic solvents as a confounding factor and as a cause of bias on the effects of CS_2. On the other hand, the persons in the first group were almost exclusively exposed to CS_2, as they were situated near by these industries.

Furthermore, majority of effluent water released by the industries in the study area was mostly contaminated with CS_2 and some of the industries release their effluents either on the open land or in surrounding surface water bodies contaminating the soil and surface water ultimately turned into groundwater. Much of the groundwater is unsuitable for irrigation, and hundreds of wells in the region are no longer in use. Based on the

earlier reports, we recruited experimental subjects who were known to live for the past 3 decades in and around our study area and were occupationally exposed to CS_2 by mode of air and water (drinking).

Occupational exposure may contribute to the development of pernicious illnesses, many times through mechanisms that involve genotoxic changes. Continuous efforts have been made to identify genotoxic agents, to determine conditions of harmful exposure and to monitor populations that are excessively exposed (Moller, 2006, Collins et al., 1997). The present study was designed to assess the genetic damage among viscose plant workers who are occupationally exposed to CS_2. CA is a valuable method for detection of occupational and environmental exposures to genotoxicants, and it can be used as a tool in risk assessment for hazard characterization (Dusinska et al., 2008; Moller, 2006) of various in vitro and in vivo studies (Valverde, et al., 1998; Rojas et al., 1996; Fairbairn et al., 1995).

An epidemiological study showed a strong correlation between CS_2 concentrations from workers in viscose rayon factory and CS_2 factory indoor air concentrations up to 64 mg/m^3 (20.5 ppm). Ghittori et al., (1998) used personal passive samplers installed in the breathing zones to measure the airborne CS_2 levels for 4 hours. Most of the indoor air samples revealed CS_2 at levels below the TLV of 31 mg/m^3.

Previous studies concluded that urinary CS_2 may be a good indicator to estimate the levels of exposure to CS_2 in the workplace. A positive correlation or urinary concentrations and indoor air levels indicated that a mean level of 15.5 µg/ CS_2/L (95% CI 13.8–17.1 µg) was excreted following exposure to CS_2 at 31 mg/m^3 current occupational exposure limit in viscose industry (Ghittori et al., 1998). Cox et al., (1998) investigated 2-thiothiazolidine-4-carboxylic acid (TTCA) concentrations as a biomarker of CS_2 exposure. The observation of CS_2 in the urine samples of workers suggests that more emphasis should be placed on workplace protection factors rather than just addressing the indoor air CS_2 concentrations. Present findings suggest us that, workers should be insisted to wear protective equipment's like respirators and follow safety precautions recommended by relevant authorities.

Certain *in vitro* experiments (Le and Fu, 1996) clearly showed that CS_2 induces CA in PBL among viscose factory workers. An increased frequency of CA in PBL was observed in the study of Le and Fu, 1996 which is in line with the results of our study. However, in group II subjects, an increase in SCE frequency was observed. A possible explanation for this may be due to smoking habits of the subjects. Because a study (Bao et al., 1996) reported no increase in SCE frequency among subjects exposed to CS_2.

The observation of higher frequencies of CA in the lymphocytes of exposed individuals agrees with the earlier reports Le and Fu 1996 regarding viscose rayon workers. These are considered S-dependent alterations, frequently observed due to human chronic exposure to chemical mutagens. In the present study, CA was observed to increase with age and exposure period in the exposed groups and based on age in the controls, though it was very low in the latter. The results of the present study also indicate a role played by age in the development of CA observed in PBL of controls.

A report on hypospermia, asthenospermia and teratospermia in young workers exposed to 40-80 mg/m^3 of CS_2 confirmed gonadal injury (Lancranjan et al., 1969). Le and Fu (1996) showed that the CS_2 induce chromosome aberration in human sperm. Numerous epidemiological reports concluded that the CS_2 is toxicant to viscose industry workers (Guidotti and Hoffman, 1999; Wang et al., 1999, Wang and Shiu, 2000). In this study, experimental subjects with smoking habits showed maximum levels of chromosome and SCE alterations when compared to respective controls, which shows that the CS_2 exposure with cigarette smoking has synergistic effect on inducing genetic damage. Chromosomal aberrations were shown to be good indicators of future risk of cancer (Hagmar et al.,1994). On the other hand genetic damages are the ultimate causes of cancer because DNA base changes can be mutagenic (Poirier, 1997). The present findings highlight the importance of investigating the genotoxicity of CS_2 on viscose plant workers occupationally exposed to this organic solvent when the smoking habit is associated, since this information provides an increased degree of identification for the positive response.

The relation between exposure to CS_2 and the induction of CA needs further investigation, and more data from larger groups of CS_2 exposed individuals from different industries are also needed to analyze the confounding effects of smoking. Similarly, as in the present study the

investigated viscose factory workers were maximum men only, further investigations are needed to collect data from women workers, in order to study the possible effect of gender on CS_2 induced apoptosis and changes in the cytogenetic end-points..

Conflicts of interest: The authors stated that no conflicts of interest.

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International Journal of
Life Sciences
International Peer Reviewed Open Access Refereed Journal

Special Issue A 11: January 2018
UGC Approved Journal No 48951

Original Article Open Access

Genetic modification of *Aspergillus niger* by induced mutagenesis for lipase enzyme production

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Manuscript details:

Available online on
http://www.ijlsci.in

ISSN: 2320-964X (Online)
ISSN: 2320-7817 (Print)

Editor: Dr. Arvind Chavhan

Cite this article as:
Bhaskaran Babitha, Shankar Sushmitha and Nagaraj Sonu S (2018) Genetic modification of *Aspergillus niger* by induced mutagenesis for lipase enzyme production , *Int. J. of. Life Sciences*, Special Issue, A11: 91-95.

ABSTRACT

The present investigation was carried out to enhance the production of lipase enzyme by genetic modification of the fungi Aspergillus niger by induced mutagenesis (UV irradiation and Ethidium bromide). Aspergillus niger strain was isolated from enriched soil sample and strains were further screened for the production of lipase using Rhodamine plate method. Strain improvement was achieved in presence of Ethidium bromide with concentration of 200µgcm^{-3} in combination with treatment under UV for 300s which was proved to be effective for enhanced lipase production. The results also indicates that chemical mutagenesis in combination with physical mutagenesis proved to be effective for stain improvement in A. niger. The selected mutant can be considered as a prospective candidate for the production of extracellular lipase for various industrial applications.

Keywords: Lipase, Aspergillus niger, induced mutagenesis, UV irradiation, Ethidium bromide.

INTRODUCTION

Lipases (triacyl glycerol acylhydrolases, EC 3.1.1.3) belonging to the class serine hydrolases catalyze hydrolysis of insoluble triacylglycerols to generate free fatty acids, mono and diacylglycerols and glycerol (Das *et al*., 2016). Lipases are ubiquitous in nature produced by animals, plants and microorganisms. However due to specificity of action, microbial lipase have been the focus of attention especially for extracellular lipases (Sheikh Abdul Hamid *et al*., 2003; Yuan *et al*., 2010).

Lipases have shown its potential in various industries such as food and dairy, detergent, cosmetic, leather, paper and pulp, biodiesel and pharmaceutical (Mohammed Rabbani *et al*., 2013, Hasan *et al*., 2007). With increasing demand for lipases in various fields in the last few

decades, serious attention towards qualitative improvement and quantitative enhancement methods are required. Quantitative enhancement includes strain improvement and medium optimization for the overproduction of the enzyme, as the quantities produced by culture strains are low (Sita Kumari Karanum *et al*., 2008). Genetic modification studies have contributed a great deal to understand the thermostability and active site mechanism for industrial improvement (Sonal Sareen Pathak *et al*., 2015). Most of the wild strains which have potential use in industrial fermentation processes are subjected to industrial strain improvement to make fermentation economical (Mishra L *et al*., 2014).

The objective of the present investigation is to isolate and enhance the lipase production by induced mutagenesis (UV irradiation and Ethidium bromide) of filamentous fungi *Aspergillus niger*.

MATERIALS AND METHODS

Sample collection and Isolation of lipase producing microorganisms

Soil samples were collected from oil contaminated site at a depth of 2-5 cm in sterile plastic bags. The soil sample was enriched for the lipase production by mixing the soil with Neem oil seed cake. Daily sprinkling of the mixture of tween 80 and olive oil over a period of 10-15 days was followed (Manjula *et al*., 2017).

For isolation of microorganisms, 10g of samples were suspended in 90 mL of sterile physiological saline solution. The solution was subjected to stirring for 30 min at 120 rpm to break the soil clumps and allowed to settle down. The supernatant was decanted and a ten-fold serial dilution method in normal saline was used to dilute the samples. Sterile Martin Rose Bengal Agar (MRBA) plate was inoculated with 100 µL of each dilution tube and incubated at 28 °C for 3-4 days. A loop of each fungal colony observed in the agar plate was sub-cultured on Potato Dextrose Agar (PDA) plate and checked for growth separately.

Morphological identification of fungal isolates

The texture and colour of the fungal colonies were observed. Fungal nature of the colonies was confirmed by lacto-phenol cotton blue staining.

Screening of lipase producing microorganisms

Rhodamine B plate assay was used for screening of lipolytic activity of isolated strains (Kouker G *et al*., 1987). The fungal isolates were inoculated on media containing 0.8% w/v nutrient broth, 0.4% w/v NaCl, 500 µL of 0.01% Rhodamine B solution, 1% w/v agar and 7.5% v/v neem oil, adjusted to pH 7.0. The plates were then incubated at 37°C for 24 h. Lipase production is detected by irradiating the plates with UV light at 350 nm. Fungal colonies that have lipolytic activity showed orange fluorescent halo.

Induced Mutagenesis

Conditions for UV Mutagenesis

The protocol described by Karanam *et* al., 2008 was adopted. The spore suspension containing 1.5 x 10^5 conidia cm^{-3}, obtained from 7 days old culture of parental strain was subjected for UV treatment. The experiment was carried out in a UV Illuminator fitted with TUP 40w Germicidal lamp which has about 90% of its radiation at 2540-2550 A°, at a distance of 16 cm away from the center of the Germicidal lamp (UV light source). The exposure time was 180s and 300s. Each UV exposed spore suspension was stored in dark overnight to avoid photo reactivation. It was then serially diluted in phosphate buffer and plated on MRBA medium. The inoculated petri dishes were incubated at 30^o C for 7 days. The survived fungal colonies were isolated as pure cultures on MRBA plates and stored at 4^oC for further use.

Conditions for Chemical Mutagenesis

The protocol described by Iftikhar *et al*., 2010 with modifications was followed. A stock of 0.5 mg mL^{-1} Ethidium bromide was prepared. A culture of fungal spores (4.63 X 10^7 spores mL^{-1}) in 9 mL of Vogel's medium was added with concentrations of 100µg cm^{-3} and 200µg cm^{-3} of ethidium bromide solution and incubated for 120s. It was then centrifuged three times at 10,000 rpm for 15 min., to remove the traces of mutagen. The experiment also included samples which were exposed to combined treatment of UV for time intervals of 180s and 300s and ethidium bromide for 120s. The treated spore suspension (0.1 mL) after different time intervals was transferred to PDA plates (4%) having 1% oxgall as colony restrictor and incubated at 30°C for 3-5 days.

Lipase enzyme extraction

The pure culture was grown on MRBA broth to obtain Mycelia mat for further process using different neem oil concentrations (500 µl and 1000 µl). After 7 days of incubation the mycelia mat (*Aspergillus niger*) in the flask was subsequently filtered by using muslin cloth. The filtered mat was homogenised with 20mM phosphate buffer and centrifugation was carried out at 10000rpm for 20min. Further the culture filtrate was used as the crude enzyme source.

Enzyme assay/ Lipase assay (titrimetric method)

The lipase activity was determined using modified titrimetric method (Manjula *et al*., 2017, Borkar *et al*., 2009). The assay mixture composed of neem oil containing 1% (v/v) tween 80 solution, 4ml of 0.1M sodium phosphate buffer (pH 7.0), 500µl of 2% calcium chloride and 1ml of enzyme filtrate. The reaction mixture was incubated at 37°C in water bath for 20min with frequent shaking for every 5-10 min. The reaction was terminated by addition of 20ml of acetone: ethanol mixture (1:1 v/v). The reaction mixture was titrated against 0.1N Sodium hydroxide. The lipase activity was calculated using the following formula:

$$\text{Lipase Activity} = \frac{(Test - Control) \times Normality\ of\ NaOH}{Incubation\ time\ (min)} \times 100$$

= ……U/ml/min.

Unit Activity:

One unit of lipase activity was defined as the amount of enzyme liberating one micro mole of fatty acid per minute under standard assay conditions.

RESULTS AND DISCUSSION

Isolation and Screening of fungal strain

Aspergillus niger strain was isolated from enriched soil sample. The strain was characterised based on their macro and micro-morphological characteristics (Fig. 1& 2). The microscopic examination carried out by lactophenol cotton blue staining procedure revealed the morphological feature of mycelia and spore characteristics. Black coloured *Aspergillus* colony possessed black conidia and black coloured spores were observed on the surface of the colony.

Strains were further screened for the production of lipase using Rhodamine plate method (Fig. 3). *Aspergillus* species exhibited extracellular lipase activity with the formation of orange fluorescent halos around the fungal colonies when it was observed under the UV light (Fig.3). In Penicillin species also Rhodamine assay was carried out to screen the lipase- producing organisms and similar results were observed (Manjula et al., 2017). Rhodamine fluorescence-based assay was used to screen 32 fungal species from diverse sources, resulted in the effective screening of fungi species (Savitha et al., 2007). The possible explanation for the formation of fluorescent products generated from trioleoylglycerol hydrolysis has been described in which rhodamine B is used in the presence of uranyl ions, yielding orange fluorescent complexes with an excitation wavelength of 350 nm. The mechanism suggested is a complex formation between cationic rhodamine B and the uranyl-fatty acid ion (MacKenzie et al., 1967). According to Kouker G et al. (1987) a conceivable mechanism may be the generation of excited dimers of rhodamine which fluoresce at longer wavelengths than the excited monomer (excimer fluorescence).

Enzyme assay/ Lipase assay (titrimetric method)

The lipase activity was carried out by estimating the free fatty acid content in blank and in the test after the incubation of neem oil substrate with enzyme by titrimetric method. To study the effect of neem oil on lipase production by *Aspergillus* sp., the fungus was grown in medium containing different concentrations of neem oil (Table 1). Lipase was found to be produced only in the presence of different oils indicating the inducible nature of the enzyme (Savitha *et al*., 2007).
The titrimetric method for determination of the enzyme lipase activity yielded as follows:

The crude extracellular lipase activity was determined as 1.80 and 2.54 U/ml/min by titrimetric method which acts as control. These values are in agreement with previous study made by Manjula *et al*., 2017 in *Penicillin sp*.

The activity of lipase enzyme under UV treated for 180 sec and 300 sec respectively was found to be 5.28 and 6.30 U/ml/min respectively. Similarly, treatment with ethidium bromide at concentrations of 100µgcm^{-3} and 200µgcm^{-3} was found to be 6.34 and 6.45 U/ml/min respectively. The combined treatment of samples with ethidium bromide and UV mutagenesis yielded better results than compared with individual treatment. Ethidium bromide with concentration of 200µgcm^{-3} in combination with treatment under UV for 300s proved to be effective for enhanced lipase production.

Table 1: Lipase activity of *Aspergillus niger* under different concentrations of Neem oil

Sl.no	Volume of substrate (Neem oil) (mL)	Source of enzyme (Condition of mutagenesis)	Activity (U/mL/min)
1	0.5	-	1.80± 0.095
2	1.0	-	2.54±0.057
3	1.0	UV treated for 180s	5.28±0.057
4	1.0	UV treated for 300s	6.30±0.011
5	1.0	EtBr 100µgcm^{-3}	6.34±0.023
6	1.0	EtBr 200µgcm^{-3}	6.45±0.020
7	1.0	EtBr 100µgcm^{-3} & UV treated for 180s	6.60±0.023
8	1.0	EtBr 100µgcm^{-3} & UV treated for 300s	6.88±0.057
9	1.0	EtBr 200µgcm^{-3} & UV treated for 180s	7.49±0.057
10	1.0	EtBr 200µgcm^{-3} & UV treated 300s	7.85±0.037

NOTE: Each value is an average of three replicates ± denotes standard deviation among replicates

Figure 1

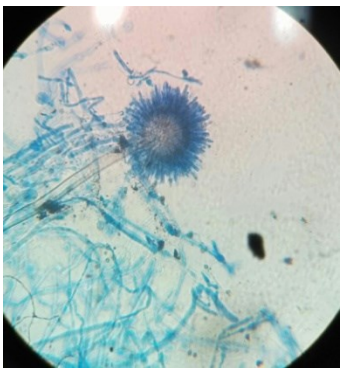

Figure 2

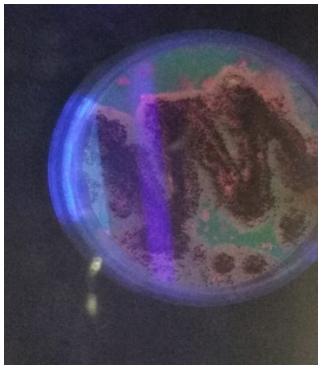

Figure 3

Figure 1: *Aspergillus niger* strain isolated from enriched soil and grown on MRBA media**.**
Figure 2: Microscopic image of *Aspergillus* sps under 40x magnification.
Figure 3: Pure culture of *Aspergillus* sps grown on rhodamine assay plate.

Improvement of microbial strains for the overproduction of industrial products has been the hallmark of all commercial fermentation processes (Karanam *et al*., 2008). The use of physical and chemical mutagenic agents such as UV, HNO_2, NTG *etc*., for hyper-production of lipase enzyme has been reported by many workers (Bapiraju *et al*., 2004; Karanam *et al*., 2008; Toscano *et al*., 2011). An increase in lipase production of 3.25-fold was reported in *Pseudomonas* mutant obtained by UV, HNO_2 and NTG (Caob and Zhanga., 2000). Lipase yield was increased to 200% in *Aspergillus niger* mutant from UV and NTG treatments (Elliah *et al*., 2002). Karanam *et al* (2008) also reported an increase in lipase production by 276% by strain improvement of indigenous isolate *A. japonicus* by UV, HNO_2 and NTG. According to Toscano *et al* (2011), NMG concentration of 200 µg cm^{-3} for 4 h resulted in enhanced lipase activity of 15.5 Ucm^{-3}. Chand *et al* (2005) used a mixture of NTG, ethidium bromide and UV or a mixture of NTG and ethidium bromide to mutate the fungal strain and found that the resultant mutant strains showed more activity in cellulase production than the wild type strains.

The molecular mechanism of genetic modification is still unclear. There are reports on epigenetics being the basis of modification (Aghcheh *et al*., 2015). Among the genes of *Aspergillus niger* where sense transcription dominated on straw but antisense predominates on glucose were several permeases, carbohydrate active enzymes, and a putative lipase (Delmas *et al*. 2012).

CONCLUSION

In industrial biotechnology, microorganisms have become a major source for the production of primary and secondary metabolites, as well as enzymes and recombinant proteins. Extensive strain improvement strategies were laid down initially by classical mutagenesis and today by advanced genetic manipulation techniques. In the present findings, *Aspergillus niger* developed as a tool for strain improvement for higher lipase production. The now emerging knowledge about how epigenetic mechanisms influence regulatory processes and the interplay of epigenetics and product formation by industrially used fungi needs to be investigated.

Acknowledgement

Financial assistance from Karnataka State Council for Science and Technology (KSCST) and Karnataka State Biofuel Development Board (KSBDB) is gratefully acknowledged. We are also thankful to Department of Biotechnology, Maharani Lakshmi Ammanni College for Women Autonomous, Malleshwaram, Bangalore for providing us laboratory facilities.

Conflicts of interest: The authors stated that no conflicts of interest.

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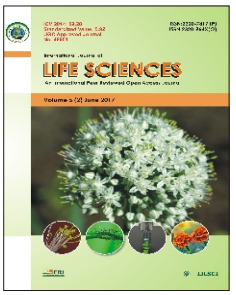

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