# Antibiotic Resistance - A Global Threat to Human Society

Siby Joseph*[1], Simi George[2], Sonal Shekhar M[3], Meera N.K[4]

[1]Department of Pharmacy Practice, Amrita School of Pharmacy, Cochin, Kerala, [2]Caritas college of Pharmacy, Kottayam,Kerala, [3]Department of Pharmacy Practice, Manipal College of Pharmaceutical Sciences, Manipal, [4]Department of Pharmacy Practice, Visveswarapura institute of pharmaceutical sciences, Bangalore Karnatak

*For Correspondence Email: sibyjoseph2007@gmail.com.

Received Date : 14.02.2012 Accepted Date : 02.04.2012

## ABSTRACT

Antibiotics, the right weapon to kill or control the growth of pathogenic bacteria could make miraculous cure for some of the untreatable or even life threatening infectious diseases till last few years. The increased number of antibiotics made the choice of antibiotics for a particular infection. This emergence could not sharpen the weapon as the microbes are clever enough to produce some alterations in their structure or activity to withstand this thread. The saddest thing regarding the antibiotics as per the one who discovers them is that due to the development of resistances most of the antibiotics become obsolete within a short span of time which is not even sufficient to meet the expenses which they already spend. This might be the reason for not having newer antibiotics in the pipeline for the forthcoming decade. So this is the time to act more seriously otherwise we will land up on a stage where there will not be any appropriate antibiotic to treat the multidrug resistant bacteria.

Keywords: Antibiotic resistance, Antimicrobials, Infection, superbugs

## INTRODUCTION

Before the term Antibiotic was described for the first time by Selman Waksman, an American microbiologist in 1942, Louis Pasteur and Robert Koch observed that an airborne bacillus could inhibit the growth of Bacillus anthraces. Although the term Antibiotic means agents that kill or retard growth of bacteria, now a days this term denotes a broader range of antimicrobial compounds including antifungals too. [1,2] The discovery of antibiotics could make miraculous cure for some of the infectious diseases which were life threatening or otherwise not treatable at all, and both the patients as well as the health care providers saluted the scientists behind the discovery of antibiotics. [3]

As the time passed more and more antibiotics came into market which made choices of antibiotics easier for treating most of the infectious diseases. Then there was a shift in the focus from the infectious diseases to life style related diseases like diabetes, hypertension, metabolic syndrome etc.[4] Even though antibiotics are being classified by different methods, cephalosporin are the most widely used class, which are sometimes grouped into generations based on their antimicrobial properties. Even though bacteria are developing resistance to fourth generation cephalosporins, we are blessed with another advanced generation of cephalosorins which we call as fifth generation cephalosporins with powerful antipseudomonal characteristics.[5]

The microbes too are clever enough to make some alterations in their structure or activity, so that they can withstand the threat caused by the emergence of variety of antibiotics. Antimicrobial resistance is the resistance developed by the microorganism towards an antimicrobial agent to which it was previously sensitive [6] The development of resistance by bacteria to antibacterial drugs is a common phenomenon and so the end of antibiotic miracle is not a new theme.[7] Emergence of resistance often reflects evolutionary processes that take place during antibacterial drug therapy. Inappropriate selection of antibiotics, under certain conditions, may result in preferential growth of resistant bacteria while growth of susceptible bacteria is inhibited by the drug.[8] Overuse of antibiotics, self-prescription of antibacterials and their use as growth promoters in agriculture are additional examples of misuse.[9] Many antibacterials are frequently prescribed to

treat symptoms or diseases that do not respond to antibacterial therapy or are likely to resolve without treatment, or incorrect or sub-optimal antibacterials are prescribed for certain bacterial infections.[10] Widespread usage of antibacterial drugs in hospitals has also been associated with increases in bacterial strains and species that no longer respond to treatment with the most common antibacterials.[11]

Antibacterial-resistant strains and species, sometimes referred to as superbugs, now contribute to the emergence of diseases which were for a while well-controlled. Cabapenems were considered as the last resort to treat some serious infections caused by Klebsiella pneumoniae. But now some of the Klebsiella species too have developed resistance to carbapenems and more sadly Klebsiella and other gram negative bacteria easily share Klebsiella pneumonia carbapenamase (KPC)-and other resistant genes across species, which would make them impermeable to all drugs. The KPC-resistant gene is found on the loops of DNA called plasmids, which are present outside the bacterial cell's chromosome. During conjugation, two cells form a bridge between them, allowing the plasmid to transfer its genes from one cell to the other. Gram negative bacteria are promiscuous as they easily exchange bits of DNA, so that a resistant gene that arises in Klebsiella, for example, quickly migrates to E.coli, acinetobacter and other gram negative species. In contrast, resistance genes in Gram positives are more likely to cluster within the species.[12]

What keeps health authorities awake at night is the possibility that Cabapenem-resistance genes will propagate, undetected to everyday maladies-such as E.Coli, which is responsible for most of the millions of urinary tract infections every year. Most of these infections still respond to Tigecycline, a newer drug and Colistin,the decade old one. Tigecycline released in 2005, was the first of a new class called glycylcyclines. As the mechanism of action of Tigecycline is different from that of other antibiotics, the development of resistance to the drug by the microorganisms has been found to be slow.[13]

New Delhi Metallo-Betalactamase (NDM-1) was first detected in a Klebsiella pneumoniae isolate from a Swedish patient of Indian origin in 2008. It was later detected in bacteria in India, Pakistan, the United Kingdom, the United States, Canada, Japan and Brazil.[14] The most common bacteria that make this enzyme are Gram negative such as Escherichia coli and Klebsiella pneumoniae, but the gene for NDM-1 can spread from one strain of bacteria to another by horizontal gene transfer.[15]

The NDM-1 enzyme was named after New Delhi, the capital city of India, as it was first described by Yong et al. in December 2009 in a Swedish national who fell ill with an antibiotic-resistant bacterial infection that he acquired in India. The infection was unsuccessfully treated in New Delhi hospital and after the patient's repatriation to Sweden, a carbapenem-resistant Klebsiella pneumoniae strain bearing the novel gene was identified. Tigecycline, which got US FDA fast track approval, has been found to be effective against the New Delhi metallo-beta-lactamase multidrug –resistant Enterobacteriaceae too. Most experts blamed the emergence of this gene on the widespread misuse of antibiotics stating that Indian doctors had not yet taken the issue of antibiotic resistance seriously and that India needed both an improved policy to control the use of antibiotics and a central registry of antibiotic-resistant infections. India's scientific fraternity, including the Union health ministry, had strongly lodged a complaint against naming it after New Delhi saying it was a ploy to put a stop to and "defame India's growing medical tourism industry". The ministry went on an overdrive to convince people that Indian hospitals were safe for foreign patients and described the study malicious propaganda.[16]

Even though, it is too late to start but still is an appreciable effort by the central health ministry of India to propose a new schedule HX for inclusion and the amendment under the drugs and cosmetics act 1945 to minimize the misuse of antibiotics .The ministry has identified a group of 90 antibiotics to be included in this restricted category of drugs. Currently, antibiotics are placed under the Schedule H of the D&C Act. The new Schedule will require doctors and chemists to retain prescriptions .Once the policy takes effect, doctors, while prescribing antibiotics will have to issue two prescriptions to every patient and one copy should be kept for a period of two years by the chemists.

## CONCLUSION

Antibiotics were producing miraculous cure for the infectious diseases till the last few years. The unethical or uncontrolled use of these drugs has led to the emergence of many antibacterial resistant strains of microorganisms which could become a deadly threat to the human community making it very difficult to treat such resistant infections. Unless the bacterial evolution slows or drug development accelerates we have only prayer in front of us to the ultimate doctor who resides above us to make miracles The antibiotic usage in any set up whether it is in the hospital or in the clinic or a community should be strictly monitored.

Most of the well established pharmaceutical companies engaged in doing research in the past are not interested in finding out newer antibiotics as the microbes can make them armless before their research products can produce a business volume which they expect. This is the time to act by controlling the use of antibiotics and proper infection control techniques. The introduction of Schedule HX by the drug authority of India if implemented properly can make a change in the uncontrolled or unethical usage of Antibiotics In addition to that the Government should give special considerations for the research on antibiotics by giving the orphan drug status or by bearing some of the expenses which a pharmaceutical firm spends for.

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RESEARCH ARTICLE

# Studying Bondo and Didiayi Populations from Orissa Using Y-Chromosomal and Mitochondrial Markers

V. Rajesh*, CSV.Ramachandra Rao

MIC College of engineering and technology,
Kanchika cherla, Krishna (Dt), Andhra Pradesh, India

*For Correspondence E-Mail: reachvalluru@gmail.com

Received Date : 10.02.2012 Accepted Date : 10.05.2012

## ABSTRACT

The Modern human originated in Africa and began their journey towards different parts of the World, mainly via southern coastal route reaching up to Australia and northern land route to populate America, Europe, and northern Asia. It has been proved that India has acted as major corridor for spreading of anatomically modern human. Further, India is known for its diversity in languages, cultures, geography and its people. Recently, it has been shown that modern Indian population have become endogamous units. Therefore, it's interesting to know the origin and affinity of all the castes, tribes and religious groups inhabiting India. Orissa is inhabited by many tribal populations. Hence, it would be interesting to reveal the origin and genetic structure of tribes living there. We have analyzed hyper variable region 1 and two coding region markers of mitochondrial DNA and three Y chromosome biallelic markers (M95-O2a, M172-j2 and M82-H1) among Bondo and Didayi tribes. Y chromosomal study reveals high frequency of Austro Asiatic-Specific o2a haplogroup (approximately 38%) followed by H1 and J2 haplogroups. Mitochondrial DNA analysis revealed the high frequency of macrohaplogroup M along with few R branches. Haplogroup M3, M5, M49 (Austro-Asiatic-Specific), M35, R6, R7, etc. were observed. Thus, we conclude that the central Indian state has Austro-Asiatic genetic affinity along with indigenous Indian signatures.

**Key words :** Austro-Asiatic, Markers, Y-chromosome, Mitochondrial, SNP

## INTRODUCTION

In molecular evolution, a haplogroup is a group of similar haplotypes that share a common ancestor having the same single nucleotide polymorphism (SNP) mutation in both haplotypes. Because a haplogroup consists of similar haplotypes, this is what makes it possible to predict a haplogroup from haplotypes. A SNP test confirms a haplogroup. Haplogroups are assigned letters of the alphabet, and refinements consist of additional number and letter combinations, for example R1b1. Y-chromosome and mitochondrial DNA haplogroups have different haplogroup designations. Haplogroups pertain to deep ancestral origins dating back thousands of years.

In human genetics, the haplogroups most commonly studied are Y-chromosome (Y-DNA) haplogroups and mitochondrial DNA (mtDNA) haplogroups, both of which can be used to define genetic populations. Y-DNA is passed solely along the patrilineal line, from father to son, while mtDNA is passed down the matrilineal line, from mother to offspring of both sexes. Neither recombines, and thus Y-DNA and mtDNA change only by chance mutation at each generation with no intermixture between parents' genetic material.

Mitochondria are small organelles that lie in the cytoplasm of eukaryotic cells, such as those of humans. Their primary purpose is to provide energy to the cell. Mitochondria are thought to be reduced descendants of symbiotic bacteria that were once free living. One indication that mitochondria were once free living is that each contains a circular DNA, called mitochondrial DNA (mtDNA), whose structure is more similar to bacteria than eukaryotic organisms (see from endosymbiotic theory). The overwhelming majority of a human's DNA is contained in the chromosomes in the nucleus of the cell, but mt DNA is an exception.

Human Y chromosomes are male-specific sex chromosomes; nearly all humans that possess a

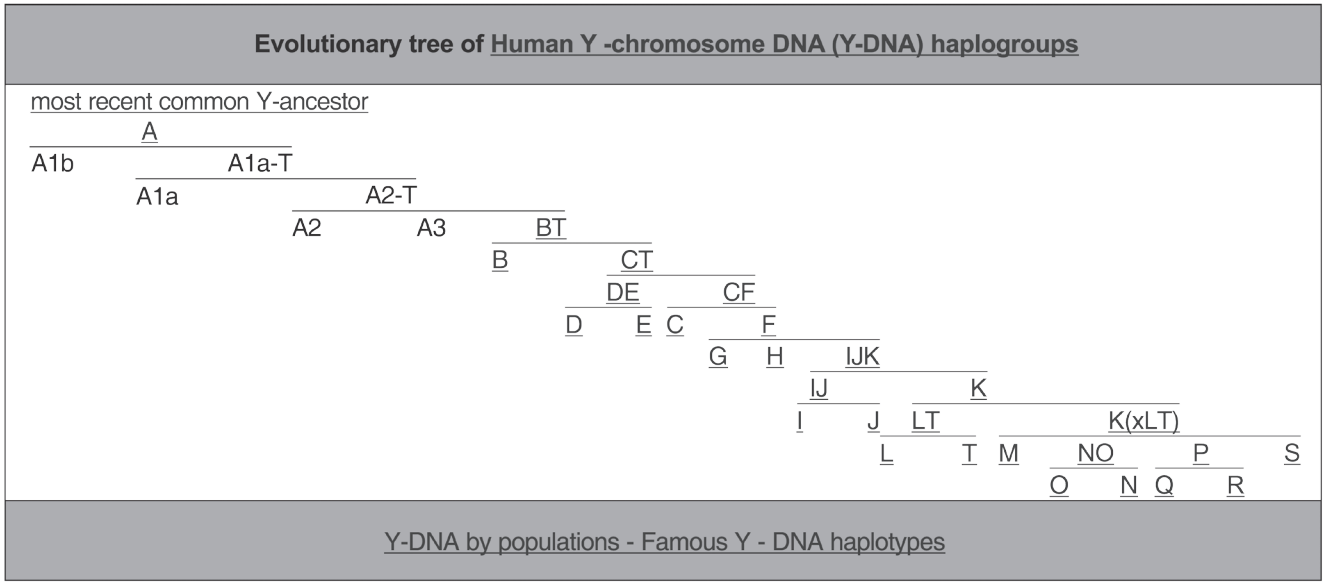
Y chromosome will be morphologically male. Y chromosomes are therefore passed from father to son; although Y chromosomes are situated in the cell nucleus, they only recombine with the X chromosome at the ends of the Y chromosome; the vast majority of the Y chromosome (95%) does not recombine. When mutations (SNPs) arise in the Y chromosome, they are passed on directly from father to son in a direct male line of descent. The Y chromosome and mtDNA therefore share specific properties.

## Human Y-chromosome DNA haplogroups

Human Y chromosome DNA (Y-DNA) haplogroups are named from A to T, and are further subdivided using numbers and lower case letters. Y chromosome haplogroup designations are established by the Y Chromosome Consortium

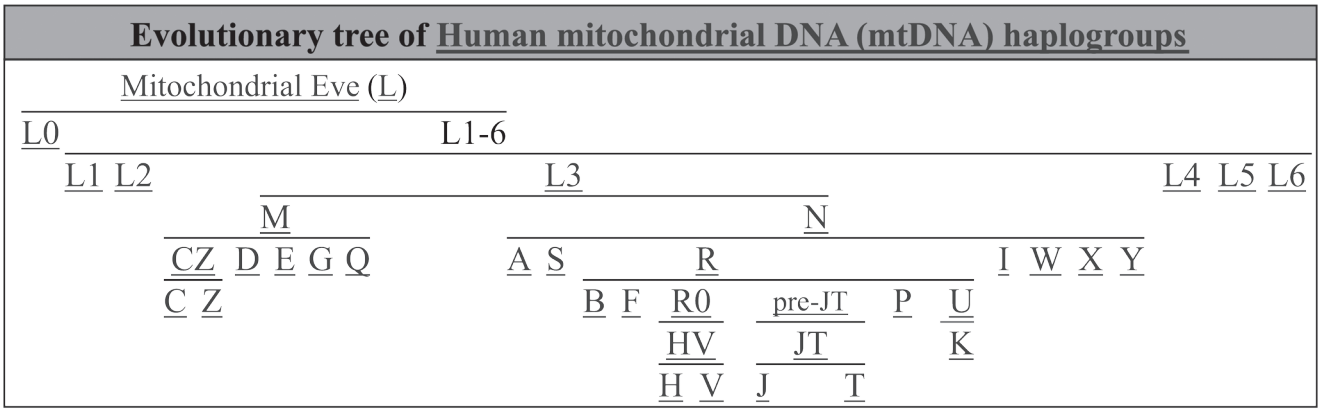
### Human mitochondrial DNA haplogroups

Human mtDNA haplogroups are lettered: A, B, C,




CZ, D, E, F, G, H, HV, I, J, pre-JT, JT, K, L0, L1, L2, L3, L4, L5, L6, M, N, P, Q, R, R0, S, T, U, V, W, X, Y, and Z.

## MATEARIALS AND METHODS



**Blood Sampling:**

10 ml of intravenous blood samples from 96 healthy and unrelated individuals belonging to Bondo&Didiayi tribe from Orissa, India was collected in vacutainers containing EDTA as an anti coagulant with their informed written concern and other pedigree details. The samples were brought to CCMB, Hyderabad in an icebox and were stored at $4^0$C until DNA was extracted from them.

**Reagents Used:**

The following solutions were prepared, sterilized and used in different experiments:

- Reagent A (Lysis buffer 1): 10mM Tris HCl (pH 8.0), 320 mM Sucrose, 5mM MgCl2, 1%Triton X.
- Reagent B (Lysis Buffer II): 400mM Tris HCl, 60mM EDTA, 150mM NaCl, 1%SDS added after autoclaving.
- Tris Saturated Phenol: Phenol, 0.1% 8-Hydroxy Quinoline, 0.5 M Tris HCl (pH 8.0), 0.1 M Tris HCl (pH 8.0).
- Chloroform: Isoamylalcohol (24:1): 24ml of Chloroform was added to 1ml Isoamylalcohol.
- Reagent C: 5M Sodium per chlorate (not to be autoclaved).
- T.E. Buffer (100ml): 10mM Tris HCl (pH 7.5), 1mM EDTA (pH 8.0).
- 20% SDS: 20g of SDS dissolved in 80ml DDW at 65°C, volume made up to 100ml.
- 70% Ethanol: 70ml of absolute alcohol in 30 ml DDW.
- 10X TAE Buffer: 48.4g Tris base, 20ml 0.5M EDTA (pH 8.0), 11.402ml glacial acetic acid, mixed and volume made up to 1 liter.
- 6x loading Dye: 0.125g of Bromophenol Blue, 0.125g of Xylene Cyanol FF, 15 ml of glycerol. Diluted with DDW to make up volume to 50ml.
- Ethidium Bromide: 10mg of Ethidium Bromide in 1 ml DDW. Stored in dark bottles.

**Reagents For PCR:**

- 10X PCR Amplification Buffer: 500mM KCl, 100mM Tris (pH 8), 15mM MgCl2, 0.1% gelatine.
- 25mM MgCl2, 2.5mM dNTPs, Primers 10ng/µl, Taq Polymerase (1 unit)

**Reagents For PCR Sequencing And Processing:**

- Big Dye ™, 50% HiDi Formamide, 70% ethanol.
- 3M Sodium Acetate: Dissolved 24.612g of Sodium Acetate in 80ml DDW, pH was adjusted to 5.2 with conc. HCl.

**Instruments Used:**

Centrifuge (Eppendorff 5810R, Biofuge, Remi R8C)

PCR machines (MJ Research PTC 200, Gene Amp 9600 Perkin Elmer, Biorad)

Electrophoresis apparatus (Pharmacia Biotech EPS600, Hoefer power pack)

Tran illuminator (Syngene)

Vortex

**Abi Prism® 3700 DNA Analyzer**

General Description: The ABI PRISM® 377 DNA Sequencer automatically analyzes DNA molecules labeled with multiple fluorescent dyes. It consists of a charge couple device (CCD) camera and a power Macintosh computer that includes software for data collection and data analysis. After samples are loaded onto the system's vertical gel, they undergo electrophoresis, laser detection, and computer analysis. Electrophoretic separation can be viewed on-screen in real-time

**Sequence Analysis Softwares:**

❖ **Sequencing Analysis Software™**

**General Description: -**

Two software packages automatically process gel files or raw sample files to analyze sample files with base calls matching sequence peaks. Sequencing Analysis Software™ Ver. 3.4.1 with free Factura™ software Ver. 2.2.2 is used for analysis of data for 310 and 377 genetic analyzers running on a Mac® OS platform. Sequencing Analysis Software™ v3.7 with Factura™ software is used for analysis of data from 310, 377, 3100 and 3700 genetic analyzers.

Both Sequencing Analysis Software™ v3.4.1 and v3.7 are powered by multiple base caller algorithms to perform signal processing and classification of peaks from raw data collected from ABI PRISM® Genetic Analyzers. The result yields accurate sequence data with electropherograms that can be viewed by Sequencing Analysis Software™ or Edit View software.

❖ **Auto Assembler version 2.0:**

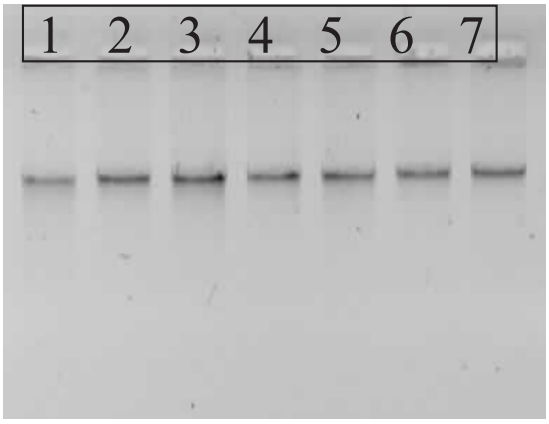
This is a sequence assembly program and can handle at least 1000 sequences of 500 bp. It allows on-screen alignment of chromatograms. The manufacturer claims that the software has no known limitations or bugs. It certainly has the nice feature of lining up all the chromatograms under each other making analysis easier.

❖ **Genedoc:**

**General Description:** A full featured multiple sequences alignment Editor, Analyzer and is Windows Based. GENEDOC has several view modes. These are accessed through the Windows menu or Project toolbar. The Alignment view, Summary View and Tree view could be opened and used at any time to get relationship among different individuals or populations.

**Methodology**

- DNA was isolated using the organic method of Gill Et.al., (1987).
- The extracted DNA was quantified by dilution check using agarose electrophoresis. (Maniatis et, al., 1989).




**Gel Image showing the bands of genomic DNA**

- PCR AMPLIFICATION OF Mitochondrial DNA (mtDNA) markers HVR1& 12F,15F

**Table 1 : Mitochondrial marker**

| S. No. | Mitochondrial Primer | Forward primer | Reverse primer |
|---|---|---|---|
| 1 | HVR I | TCATTGGACAAGTAGCATCC | GAGTGGTTAATAGGGTGATAG |
| 2 | 12F | CACCATTCTCCGTGAAATCA | AGGCTAAGCGTTTTGAGCTG |
| 3 | 15F | TCTCCATCTATTGATGAGGGTCT | AATTAGGCTGTGGGTGGTTG |

**Table 2 : PCR 'REACTION MIX' for the mitochondrial marker used**

| Mitochondrial markers | Buffer (µl) | Mgcl2 (µl) | dNTP's 2.5mM (µl) | Forward Primer 10pM (µl) | Reverse Primer 10pM (µl) | Taq (µl) | DDW (µl) |
|---|---|---|---|---|---|---|---|
| HVR I | 1.0 | 0.8 | 0.6 | 0.1 | 0.1 | 1.0 | 5.40 |
| 15F | 1.0 | 0.8 | 0.6 | 0.06 | 0.06 | 1.0 | 5.48 |
| 12F | 1.0 | 0.8 | 0.6 | 0.1 | 0.1 | 1.0 | 5.40 |

**Table: 3: PCR cycle for the mitochondrial marker used**

| Mitocho-ndrial marker | Initial Denaturation | | Denaturation | | Annealing | | Extension | | | Final Extension | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Temp. | Time | Temp. | Time | Temp. | Time | Temp. | Time | Cycles | Temp. | Time |
| HVR I | 95˚C | 5’ | 94˚C | 30’’ | 58˚C | 30’’ | 72˚C | 2’ | 35 | 72˚C | 7’ |
| 12F | 95˚C | 5’ | 94˚C | 1’ | 52˚C | 30’’ | 72˚C | 2’ | 35 | 72˚C | 7’ |
| 15F | 95˚C | 5’ | 94˚C | 30’’ | 52˚C | 45’’ | 72˚C | 1’ | 35 | 72˚C | 10’’ |

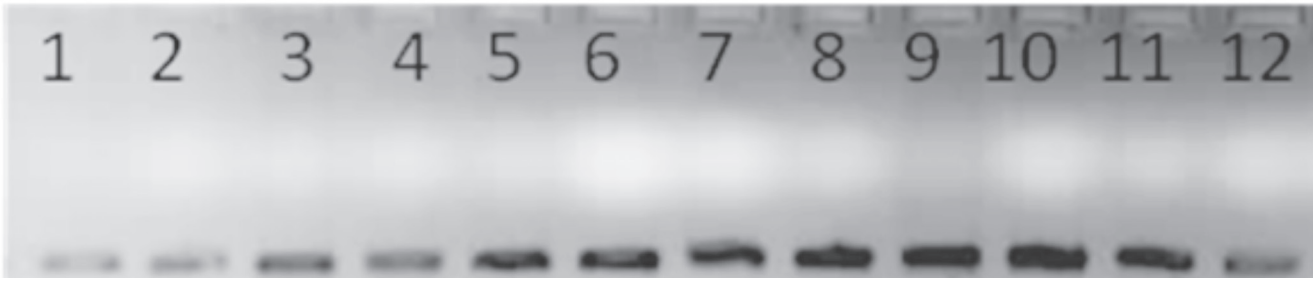



**Figure. 1 : Gel Image showing the bands of HVR1 PCR products**

- PCR AMPLIFICATION OF Y-Chromosomal DNA (Y-DNA) markers M95& M172,M82

**Table 4 : Different YSNP markers and their nucleotide substitution positions.**

| PRIMER | REGION | SIZE (bp) | SITE | MUTATION | FORWARD PRIMER | REVERSE PRIMER |
|---|---|---|---|---|---|---|
| M82 | B9.t18 | 328 | 179 | -2bp | CTGTACTCCTGGGTAGCCTGT | GAACGATTGAACACACTAACTC |
| M95 | 50f2E | 850 | 419 | G->A | TGGATTGCATTCAACTTCACTTAC | CTGAGTTCAAATGCTCGGGTCTC |
| M172 | DFFRY | 345 | 197 | T->G | TTGAAGTTACTTTTATAATCTAATGCTT | ATTTATTACTTTACAGTCACAGTGG |

- **Table 5 : PCR ‘reaction mix’ for the Y-chromosomal SNP markers used**

| Y-SNP MARKERS | Buffer (10 X) | Mgcl2 25mM | DNTP’s 2.5mM | Forward Primer 10pM | Reverse Primer 10pM | Taq | DDW |
|---|---|---|---|---|---|---|---|
| M95 | 1µl | 0.8µl | 0.6µl | 0.06µl | 0.06µl | 1µl | 5.48µl |
| M82 | 1µl | 0.8µl | 0.1 µl | 0.1 µl | 0.1 µl | 1µl | 5.40µl |
| M172 | 1µl | 0.8µl | 0.6µl | 0.1µl | 0.1µl | 1µl | 5.40µl |

**Table 6 : PCR conditions for the Y-chromosomal SNP markers used**

| Y-Chromosomal marker | Initial Denaturation | | Denaturation | | Annealing | | Extension | | | Final Extension | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Temp | Time | Temp | Time | Temp | Time | Temp | Time | Cycle | Temp | Time |
| **M95** | 95˚C | 5’ | 94˚C | 30’’ | 58˚C | 30’’ | 72˚C | 2’ | 35 | 72˚C | 7’ |
| **M82** | 96˚C | 5’ | 94˚C | 30’’ | 54˚C | 30’’ | 72˚C | 2’ | 35 | 72˚C | 7’ |
| **M172** | 94˚C | 5’ | 94˚C | 30’’ | 55˚C | 30’’ | 72˚C | 2’ | 35 | 72˚C | 7’ |

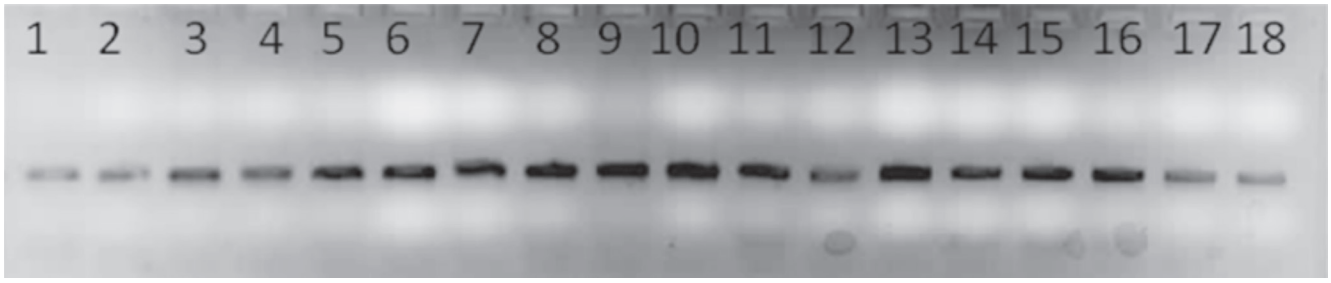
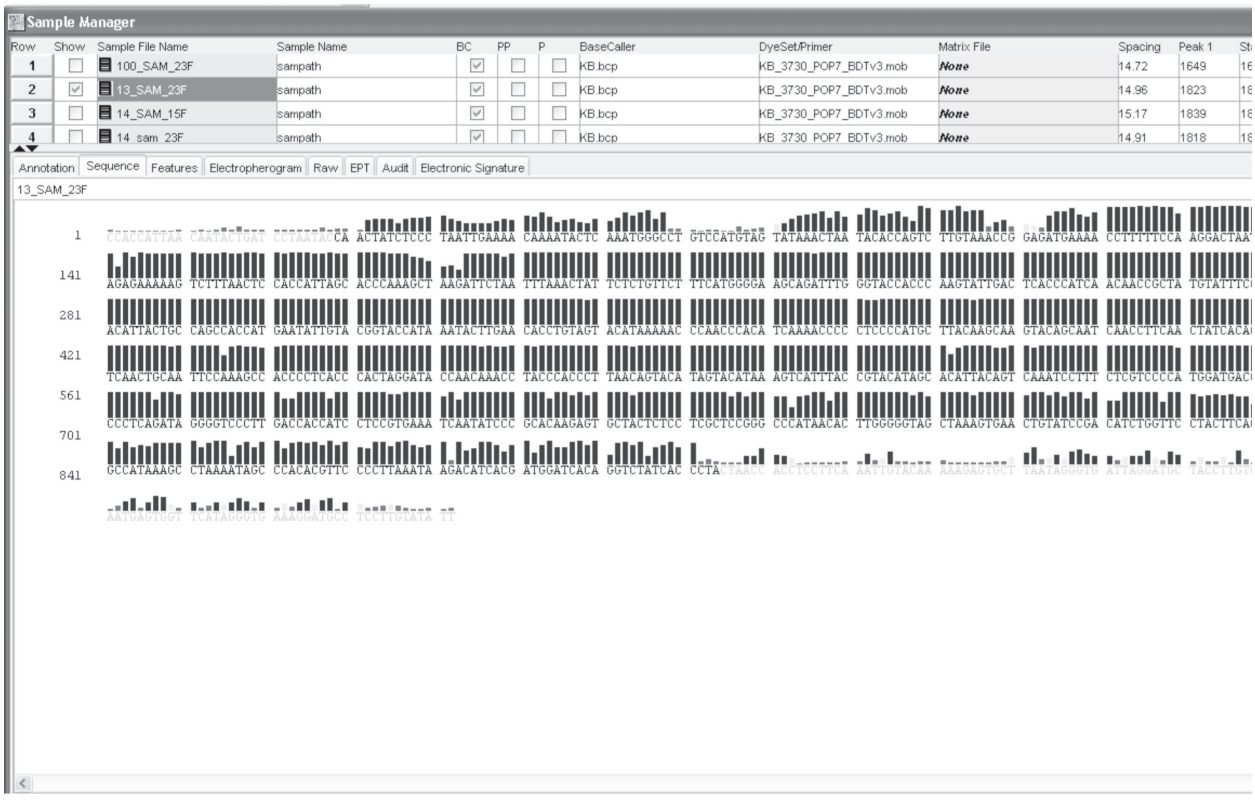



**Figure. 2 : Gel Image showing the bands of M95 PCR products**

- PCR products of HVSI (mtDNA -D loop) and Y-Chromosomal markers (SNPs) respectively were directly sequenced using the ABI Prism 3700 DNA analyzer.



## RESULTS AND DISCUSSIONS

Bondo and Didiayi Tribal population from Malkangiri & Kudumulgumma region of Orissa was taken for the study. It was analyzed using mitochondrial and Y chromosomal markers and the haplogrouping results obtained was discussed below.

With the exception of the diverse set of largely Indian-specific R lineages, the most frequent mtDNA haplogroup in India that derives from the phylogenetic node N is haplogroup W (Kivisild et al. 1999a; Kivisild et al. 1999b). The frequency peak of haplogroup W is (approximately 1%) in the north-western states. Elsewhere in India its frequency is very low (from 0 to 0.9%) forming a significant spatial cline (Metspalu et al. 2004).

The most frequent sub-clade of R in India is haplogroup M, with Indian-specific variants of M5(a)(Kivisild et al. 1999a; Quintana-Murci et al. 2004). Haplogroup M, mtDNA lineage cluster with an estimated age of 51,000–67,000 years, represents the most profound overlap between western-Eurasian and Indian mtDNA lineages. The frequency of haplogroup M in India reaches 15% among the caste and 8% among the tribal populations (Kivisild et al. 1999a; Kivisild et al. 2000; Metspalu et al. 2004).

The most common sub-cluster of M in India are Indian-specific clades of M (m3,m33a1,35,m41,m45,m49d), which do not show a distinct geographic cline and are present throughout India and coalesce with western-Eurasian M lineages 53,000 ± 4,000 ybb (Kivisild et al. 1999a; Quintana-Murci et al. 2004); Metspalu 2004).

Other subset of haplogroups B5, G2a2, X2a are present with a frequencies of (approximately 1%,1%,3%). This is evident from the high frequencies of these haplogroups in Gujarat and iran and the haplotypes present in those localities (Metspalu et al . 2004). Over two third of western Eurasian specific maternal lineages in the Indian mt DNA pool belongs HV, The most frequent haplogroup is uncertain haplogroup UV (approximately 64%) (Kivisild et al. 1999a; Metspalu et al. 2004)

## CONCLUSION

The objective of this project was to infer about the genetic diversity of two tribe populations of Orissa region with other populations of India. In the overall analysis, it was observed that most of the individuals of all two tribe population were falling in Indian specific macrohaplogroup M. Further, in present study, we have found high percentage of Indo-European haplogroups (21%) which suggest interestingly the strong influence of Eastern wave. The mitochondrial 9 bp deletion is found in 1out of 96 samples analyzed.

In addition Y chromosomal analysis is showing approximately 38% percentage of The Indo European signature M95-o2a was found very prominent.

Also it is evident that our investigation of the small population can offer no more than snapshot of Indian pre history from the genetic perspective .In future detailed phylogeographic and phylogenetic analyses of more tribal population can reveal some interesting patterns of maternal as well as paternal lineages and genetic footprints of India population.

Recent studies by Thangaraj et.al. 2005a, b opens new insights to many unique studies that can be made to found unique patterns of genetic foot prints of different maternal and paternal lineages in India.

## ACKNOWLEDGEMENT

The authors are grateful to Dr.k.Thangaraj Scientis E and Dr. Ch Mohan Rao, Director; CCMB Hyderabad Who gave me an opportunity to practical training in one of the well equipped & prestigious Molecular biology labs in our country

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# *In-Vitro* and *In-Vivo* Study of Indomethacin Transdermal Patches

V.V.Venkatachalam[1], S.Ravichandran[2]

[1]Department of Pharmacy, Annamalai University, Annamalainagar.

[2]Department of Pharmaceutical Analysis, PSG College of Pharmacy, Coimbatore.

*For correspontance e-mail: bsravichandran@yahoo.co.in

Received Date : 29.04.2012 Accepted Date : 31.05.2012

## ABSTRACT

To treat arthritis and inflammatory disorders on long term therapy needs plasma concentration of drug in better manner. This was achieved by formulating the drug in controlled release pattern. Indomethacin is the non Cox selective NSAID available in market and formulated in transdermal drug delivery system for controlled release of drug from matrix type of patch. In-vitro release study of Indomethacin transdermal patch of Formulation F11 shown release of drug 97.88 % at 24 h and also follows zero order kinetics release pattern. In-vivo study on rabbits of Formulation F11 proves that drug release was 96.2 % at 24 h.

**Key words :** Indomethacin, patch, In-vitro, In-vivo.

## INTRODUCTION

Indomethacin is a non Cox selective category of NSAID available in the market. Arthritis and inflammatory disorders are difficult task for public health concern in so many countries. Indomethacin is used in the treatment and management of arthritis and inflammatory conditions with the recommended dose of 25 mg thrice a day. But management of inflammatory conditions need the blood concentration of drug in a steady manner for better results, so alternate route of administration is adopted by preparing a transdermal therapeutic system of Indomethacin

## MATERIALS AND METHODS :

Indomethacin , HPMC K-10, PVP K-30, EC 14cps, ethanol, DBP were purchased. All other chemicals used were of analytical grade.

### Preparation of matrix patches:

Polymers of ethyl cellulose, hydroxy propyl methyl cellulose and polyvinyl pyrolidine were accurately weighed and dissolved individually or in combination in 5 ml of ethanol. The drug was then dispersed in the polymeric solution and then a plasticizer of dibutyl phthalate was added. The solution was stirred to attain semisolid like consistency and casted on a glass substrate containing 'o' ring, the rate of evaporation of solvent from polymeric solution was controlled by placing an inverted funnel at room temperature for a day[1,2,3,4]. The formed films were separated. Formulation of Indomethacin was given in table. no: 1.

### Preparation of rate controlling membrane :

Ethyl cellulose 1% W/V was dissolved in ethanol of 5 ml, to this plasticizer of dibutyl phthalate was added, the solution was mixed to get a semisolid like consistency and casted on a glass substrate containing 'o' ring, the rate of evaporation of solvent from polymeric solution was controlled by placing an inverted funnel at room temperature for a day. The drug contained patch was fixed with a rate controlling membrane by ethanol, then wrapped in an aluminium foil and stored in a dessicator [5].

### In-vitro release study :

The prepared Indomethacin patch was evaluated for release pattern using commercially available semi

permeable membrane. The membrane and patch were fitted between donor & receptor compartment of self fabricated modified Franz diffusion cell [6]. The donor compartment was empty & receptor compartment was containing 50 ml of phosphate buffer pH 7.2. The samples were collected at different time intervals for analyzing the drug content in the receptor compartment for release pattern of drug and replaced with equal volume of freshly prepared phosphate buffer pH 7.2. The drug content was analyzed at 319 nm using U.V double beam spectrophotometer (table.no:2). From the study best formulation was selected for further studies.

**In-vivo study :**

The formulation F11 was used for in-vivo study on rabbits, rabbits were selected and its hair was removed from dorsal surface with scissor. Animal dose was calculated as 2.11 mg and the patch size was reduced to 1 cm2 to apply on the rabbit also drug free patch was formulated for control study group. Different group of animals were categorized and the patch was applied at same time to all the groups, but removed at different time intervals (table. no:3). The formulation F11 was studied for in-vivo drug release in rabbit model using remaining drug content formula 7, in that method patch was applied and removed at particular time and then analyzed for drug content at 319 nm U.V spectrophotometer8.

$$\text{Drug in blood} = \frac{\text{Amount of drug in patch before placing} - \text{Amount of drug remaining in patch after removal}}{\text{Amount of drug loaded in patch}}$$

**RESULTS & DISCUSSION:**

The prepared Indomethacin transdermal patch was evaluated for in-vitro release pattern. The formulation F7 (EC- 0.5%, PVP-0.5%) shown release of 89.21 % at 14 hours and F6 (EC-0.75%, PVP-0.25%) shown release of 84.99% at 15 hours. The formulation F8 (HPMC-0.33%, EC-0.33%, PVP-0.33%), showed a release of 86.70% at 16 hours. The release were not upto expected level because of less concentration of PVP hence, drug was not released fully. The formulations F1 (HPMC-1%), F4 (EC-1%), showed a release of 80.53 %, 86.89% respectively at 16 hours and formulations F2 (HPMC-2%), F3 (HPMC-3%) F5 (EC-2%) showed a release of 81.10% at 18 hours, 85.55% at 20 hours 81.22% at 20 hours respectively. It has shown that single polymer of hydrophillic (or) hydrophobic does not release drug completely. The formulation F9 (HPMC-2%, PVP-1%) showed a release of 97.23% at 19 hours. The release was 97.23% because of high concentration of PVP, but release of drug completed at 19 hours. The formulation F10 (HPMC-3%, PVP-1%) showed a release of 99.22% at 16 hours. The release was high because of high concentration of PVP, but release of drug completed at 16 hours. Because of 99.22% release, it was selected for further formulation development. To develop the formulation for 24 hours the release rate of drug to be controlled by rate controlling membrane. The formulation F11 (HPMC -3%, PVP -1% WITH EC – 1% AS RATE CONTROLLING MEMBRANE) shown release of 97.88% at 24 hours, because of PVP the release was upto the expected level and rate of release of drug extended upto 24 hours due to retardation of release by ethyl cellulose as rate controlling membrane. In-vivo study on rabbit confirms the release of drug Indomethacin in transdermal patch as controlled delivery over 24 h as 96.2 %.

**CONCLUSION:**

The drug selected as Indomethacin for transdermal therapeutic system of anti-inflammatory study showed appropriate release in both in-vitro & in-vivo studies. This confirms that the formulation F11 may control the arthritis and inflammatory disorder in better manner by achieving drug concentration in steady manner for over a day.

**Table. No 1 : Formulation of Indomethacin Transdermal Patches**

| CODE | HPMC (%) | EC (%) | PVP (%) | RCM (EC) (%) | PLASTICIZER | SOLVENT |
|---|---|---|---|---|---|---|
| F1 | 1 | - | - | - | 30% W/W of Dibutyl Phthalate | 5 Ml of 95% Ethanol |
| F2 | 2 | - | - | - | | |
| F3 | 3 | - | - | - | | |
| F4 | - | 1 | - | - | | |
| F5 | - | 2 | - | - | | |
| F6 | - | 0.75 | 0.25 | - | | |
| F7 | - | 0.5 | 0.5 | - | | |
| F8 | 0.33 | 0.33 | 0.33 | - | | |
| F9 | 2 | - | 1 | - | | |
| F10 | 3 | - | 1 | - | | |
| F11 | 3 | - | 1 | 1 | | |
| Each formulation contains 30mg of INDOMETHACIN | | | | | | |

**Table.No 2 : In-Vitro Release of Indomethacin Transdermal Patches**

| FORMULATION CODE | CUMULATIVE PERCENTAGE OF RELEASE | TIME OF RELEASE |
|---|---|---|
| F 1 | 80.53% | 16 hrs |
| F 2 | 81.10% | 18 hrs |
| F 3 | 85.55% | 20 hrs |
| F 4 | 86.89% | 16 hrs |
| F 5 | 81.22% | 20 hrs |
| F 6 | 84.99% | 15 hrs |
| F 7 | 89.21% | 14 hrs |
| F 8 | 86.87% | 16 hrs |
| F9 | 97.00% | 19 hrs |
| F10 | 99.22% | 16 hrs |
| F11 | 97.88% | 24 hrs |

**Table.No 3 : In-Vivo Study of Fexofenadine Hydrochloride Transdermal Patches**

| TIME (hrs) | AMOUNT OF DRUG RELEASE (mg) | PERCENTAGE OF DRUG RELEASE (%) |
|---|---|---|
| 6 | 0.49 | 24.29% |
| 12 | 1.03 | 51.21% |
| 18 | 1.55 | 77.95% |
| 24 | 2.03 | 96.20% |

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RESEARCH ARTICLE

# Evaluation of Treatment Outcomes in Rheumatoid Arthritis: a Prospective Study

Premkumar B*[1], Srinivasa Murthy M [2], Rajagopal K [3]
[1]PSG College of Pharmacy, Peelamedu, Coimbatore, Tamilnadu
[2]Vignan Institute of Pharmaceutical Sciences, Vignan Hills, Nalgonda, A.P, India
[3]Aizant drug research solutions private Ltd, Hyderabad, A.P, India

Received Date : 11.06.2012 Accepted Date : 20.07.2012

## ABSTRACT

The objective of the study was to evaluate the treatment outcomes of different treatment regimens followed for rheumatoid arthritis (RA) patients. Based on the prescription pattern, the treatments were grouped into four categories. Disease Modifying Anti-Rheumatic Drugs (DMARD) was present in all the cases. The clinical benefits were evaluated for 18 weeks, therapeutic efficacy assessed based on the Visual Analog Scales for pain, Patient's and Physician's global assessment and fatigue. Morning stiffness, Erythrocyte Sedimentation Rate (ESR) and the disease activity were measured by means of self-reported Health Assessment Questionnaire (HAQ). At the end of baseline visit 57 patients with Rheumatoid Arthritis enrolled in the study, and 40 patients completed the study. Female preponderance was observed and the disease duration was around 3 years. With regard to patient reported outcomes, the group with triple combination of DMARD, steroid and NSAID was found to be superior when compared to all the other groups. The NSAID combinations proved to be beneficial in alleviating the symptomatic features of the disease such as pain. In comparison with other groups, addition of steroids to DMARDs and NSAIDs had resulted in clinically relevant improvement at several assessment points. The clinical results favor the DMARD with steroid and NSAID might have synergistic effects, acting at different pathways of inflammatory cascade. The study confirms that highly effective control of disease activity can be achieved using conventional DMARDs and their combinations, as a part of intensive management strategy.

**Key words :** Rheumatoid Arthritis, DMARD, Steroid, NSAID, Health Assessment Questionnaire, Visual Analog Scale

## INTRODUCTION

Rheumatoid arthritis (RA) is the most common form of inflammatory arthritis affecting nearly one in 100 adults. RA is traditionally considered as a chronic, inflammatory autoimmune disorder that causes the immune system to attack the joints. It is a disabling and painful inflammatory condition, which can lead to substantial loss of mobility due to pain and joint destruction. Rheumatoid arthritis has a detrimental effect on many areas of life, including physical, psychological and social functioning. RA is a systemic disease, often affecting extra articular tissues throughout the body including the skin, blood vessels, heart, lungs and muscles. 1-4The exacerbation of the disease peaks at only certain times of the day and the cardinal symptoms of rheumatoid arthritis include: Stiffness, swelling and pain of one or more joints of the body characteristically severe in the morning, fatigue and weakness. DMARDs have been found to produce durable remissions and delay or halt disease progression. They prevent bone and joint damage and are preferred first line drugs. These drugs have several adverse effects, intolerance which leads to discontinuation of treatment. The practice of combination therapy has received general acceptance. There are many unresolved issues such as best practice regimen and studies are needed to establish accurate methods of predicting prognosis of RA. 5 Hence, the study was designed to evaluate the treatment outcomes of different combinations used in treatment of RA.

## METHODS

### Study design and patients

The study was conducted in orthopedic unit of PSG Hospital, Coimbatore from July 2012 – December 2012. The trial was designed as an 18-week, open-label, non-randomized study. The study was approved by Institutional Ethics Committee (IEC) of PSG Institute of Medical Sciences and Research. All patients gave their written, informed consent prior to participation. Men and women with age > 18 years were included. The exclusion criteria were history of malignancy, history of seizure disorder, active psychiatric disorder, major organ dysfunction, or local or systemic infection. Female patients were excluded if they were pregnant or breast feeding.

**Table 1 : Patients Characteristics and Disposition**

| | DMARD | DMARD+STEROIDS | DMARD+NSAID D | MARD+STEROID+NSAID |
|---|---|---|---|---|
| AGE (Mean±SEM) | 48.63±3.85 | 39.83±3.41 | 36.67±3.39 | 35±2.95 |
| Duration of RA (Mean±SEM) | 2.75±0.37 | 3.0±0.39 | 3.1±0.35 | 2.7±0.37 |
| Total number of patients (n) | 8 | 12 | 10 | 10 |
| Male | 2 | 1 | 1 | 3 |
| Female | 6 | 11 | 9 | 7 |
| RF positive | 4 | 7 | 5 | 8 |
| RF seronegative | 4 | 5 | 5 | 2 |

**Table 2 - Changes in ESR at baseline, 9th week and 18th week**

| Treatment | 0 week | 9 week | 18 week |
|---|---|---|---|
| 1 DMARD | 63.13±7.00 | 54.00±7.30 | 51.83±7.17 |
| 2 DMARD+1 Steroid | 61.50±4.46 | 29.83±4.94 *** | 21.50±5.14 *** |
| 1 DMARD + 1 Steroid | 51.50±4.22 | 46.80±4.21 | 43.80±4.21 |
| 1 DMARD +1 Steroid + NSAID | 51.50±4.22 | 41.00±4.06 | 30.40±3.97 ** |

*indicates comparison with baseline (** p<0.01, ***p<0.001)

### Prescription practices of orthopaedicians in the treatment of RA

The prescription pattern of orthopaedicians was recorded. At the baseline visit after analyzing the treatment pattern, the groupings were done. Based on the groupings the efficacies were compared at different intervals.

### Efficacy measurements

**VAS assessment:** Pain, Fatigue, Physician's and Patient's global assessment of the disease were analyzed with VAS (0-100mm, with higher scores indicating a greater degree of severity: 0 = none and 100 = higher). All the outcomes were measured at baseline and at follow-up visits.

**Morning Stiffness:**

The duration of morning stiffness on the day of examination should be determined by asking the following two questions. When did you awaken this morning? When were you able to resume your normal activities without stiffness? Duration of morning stiffness is equal to the time elapsed between the above two times in minutes.

**Health Assessment Questionnaire:**

The disability section of the full HAQ-DI includes 20 questions to assess physical functions in 8 domains: dressing, arising, eating, walking, hygiene, reach, grip and common activities. The questions are

evaluated on a 4-point scale: 0=without any difficulty, 1=with some difficulty, 2=with much difficulty and 3=unable to do. Higher scores=greater dysfunction. A disability index was calculated by summing the worst scores in each domain and dividing by the number of domains answered.

**Laboratory m easurements:**

Erythrocyte Sedimentation Rate (ESR) was measured as an index of status of the disease at all the visits. Rheumatoid Factor (RF) was measured at baseline.

Safety evaluation General clinical safety and tolerability were monitored throughout the study

**STATISTICAL ANALYSIS**

Data are expressed as Mean±SEM. The difference between the randomized groups was evaluated by One-way ANOVA followed by Student's t-test (post hoc Bornferroni test). All the values of <0.05 was considered to indicate statistical significance. Statistical analysis was performed using Graph pad prism software, version 1.04.

**RESULTS**

**Patient characteristics and disposition**

The demographic details were shown in (Table 1) After screening 72 patients, 57 patients were enrolled in the study and 70% completed the 18week study period. The study population comprised of 17.5% men and 82.5% women, aged 23-67years.12 drop-outs occurred due to protocol violations. Baseline characteristics were reasonably well balanced across the treatment groups.

**Prescription pattern**

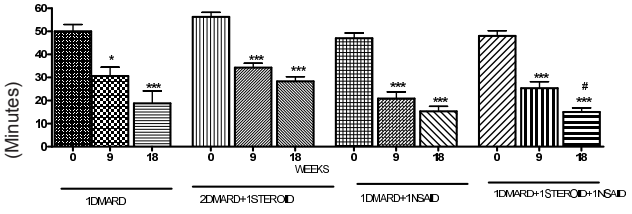
Based on the prescription analysis at baseline, it revealed the following treatment regimens such as 11 patients were on DMARDs alone (1 DMARD), 12 patients were on DMARD combination with steroids (2 DMARD+1Steroid), 14 patients were on DMARD combination with NSAID (1 DMARD+1 NSAID), and 10 patients were on DMARD combination with steroids and NSAID (1 DMARD+ 1 Steroid + 1 NSAID). 75% of the prescriptions revealed Hydroxychloroquine at 200mg dose twice daily. 37.5% of them were on Methotrexate (MTX) 7.5mg-15mg per week. All the patients on methotrexate were prescribed 10mg folic acid per week and taken on the day in which MTX was not taken. 37.5% were on Sulfasalazine 500mg twice daily. 30% were on Prednisolone at 5mg twice daily. 30% were on Aceclofenac 100mg twice daily. 20% were on Etoricoxib 90mg once daily. 5 patients were on NSAIDs alone or Tramadol with acetaminophen, but not considered for evaluation since the number of patients were very less.

**Clinical efficacy**

**Morning stiffness**

The course of change in morning stiffness is shown in the Figure (1). All the treatment groups had reduced the morning stiffness significantly. The DMARD combination with steroids and NSAID was found to be more effective at each follow-up visit. The reduction in morning stiffness corresponds to 39% (1DMARD group and 2 DMARD with steroids), 56% (1DMARD with NSAID), and 47% (1DMARD + STEROID+ NSAID) at 9th week and 53%, 50%, 67% and 69% reduction at 18th week.

**FIGURE - 1 MORNING STIFFNESS**

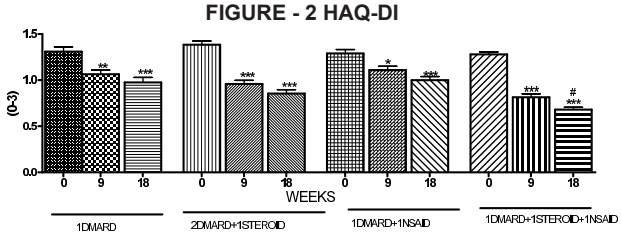



* indicates comparison with baseline (* p<0.05, ***p<0.001)
#indicates comparison with 9th week (#p<0.05)

**HAQ-DI**

The changes in the disease index were shown in Figure (2). An improvement in functional ability, as measured by HAQ was seen at all the follow-up visits in the steroids group and steroids with NSAIDs. The improvements were 21% (1DMARD), 30% (2DMARD with steroids), 14% (1DMARD with NSAID), and 36% (1DMARD + STEROID+ NSAID) at 9th week and 27%, 38%, 23% and 47% improvement at 18th week.

**FIGURE - 2 HAQ-DI**

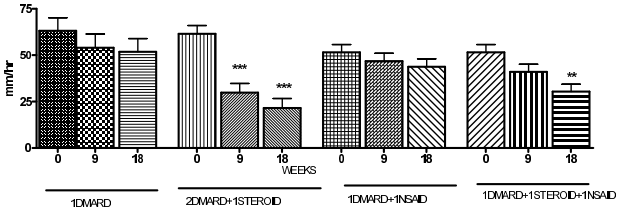



* indicates comparison with baseline (* p<0.05, ** p<0.01, ***p<0.001)
#indicates comparison with 9th week (#p<0.05

### ESR and RF

ESR changes at each visit are shown in Table (2). Improvements in mean ESR level were observed in all the groups, but more prominent results were observed in DMARD combination with steroids. The reduction were 14% (1DMARD), 51% (2DMARD with steroids), 9% (1DMARD with NSAID), and 20% (1DMARD + STEROID+ NSAID) at 9th week and 18%, 65%, 15% and 41% at 18th week. Rheumatoid Factor was positive for 25 patients and 15 patients were seronegative.

**FIGURE - 3 ESR**



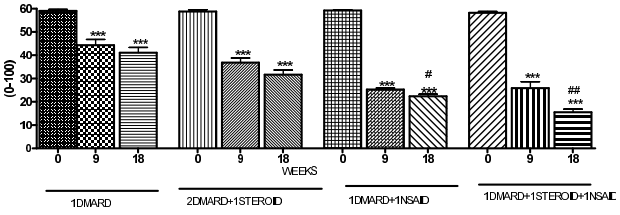

* indicates comparison with baseline (** p<0.01, ***p<0.001)

### VAS measurements

Changes in pain parameter are shown in Figure (3) Pain reduced in all the groups, but the NSAID group and the steroids with NSAID group had shown prominent relief. The reduction were 25% (1DMARD), 37% (2DMARD with steroids), 57% (1DMARD with NSAID), and 56% (1DMARD + STEROID+ NSAID) at 9th week and 30%, 46%, 62% and 73% at 18th week. Fatigue improvement was more significant in the DMARD combination with steroid and NSAID group. These details were shown in Figure (4).

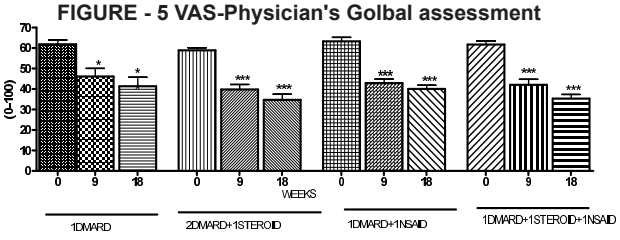
**Safety** No adverse effects were reported in this study.

**FIGURE - 4 VAS PAIN**



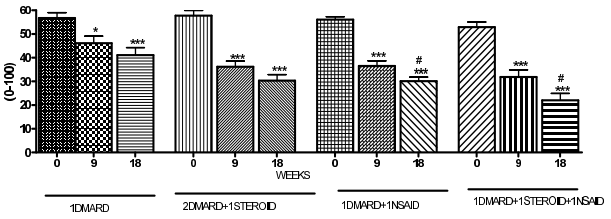

* indicates comparison with baseline (***p<0.001)
#indicates comparison with 9th week (#p<0.05, ##p<0.01)

**FIGURE - 5 VAS-Physician's Golbal assessment**




* indicates comparison with baseline (* p<0.05, ***p<0.001)

**FIGURE - 7 VAS-FATIGUE**




* indicates comparison with baseline (* p<0.05, ***p<0.001)
#indicates comparison with 9th week (#p<0.05)

## DISCUSSION

It is mandatory for the health professionals to assess the patient's needs for health care, and matching these needs is a complex task. With respect to chronic diseases, awareness of patient's preferences gets priority in healthcare. Though, the questionnaire based self-reported outcomes has limitations such as variance, self-reported questionnaires have been used as valid assessments for many years. This data may be clinically useful information and many studies have proven this.[6] The present study offers important information regarding the outcomes of different treatment regimens in the treatment of rheumatoid arthritis.

The study reveals that the practitioners still adhere to cost -effective therapies, since no newer DMARDs like leflunomide, or biologics or TNF inhibitors were not found in the prescriptions which is in concordance with previous reports.[7]

The combinations of DMARDs were widely accepted because multiple drugs act synergistically and have sustained efficacy without increase in drug toxicity. Empirically most of the practitioners use different combinations of DMARDs and this study revealed parallel strategy in contrast to step-up or step-down strategy, where a drug is added or removed.

Previous findings support the combination with low-dose steroids is more efficacious, in inflammatory disorders.[8] The steroids combination with DMARDs had proven to be effective in reducing the symptomatic aspects of the disease such as patient reported outcomes. There is strong evidence that glucocorticoids, block the release of several inflammatory mediators and a strong alleviator of stress, because of its action on the Hypothalamus-Pitutary-Axis. This reflected in the study as improvements in the fatigue, reduction in pain parameters.

Most people with rheumatoid arthritis are faced with frequent or ongoing pain and depression. Pain is an indicator of the RA disease. However, it contains both physical and psychological elements. So pain has to be given more attention and its reduction is most important clinical outcome of the disease. To alleviate pain, the best choice drugs are NSAIDs. They are not only advantageous, but in combination therapies, can act faster when compared to the DMARDs. The present study report also concludes that NSAID combinations with steroids and DMARDs are more effective in controlling the symptomatic features of rheumatoid arthritis. Since gastrointestinal disturbances are of a concern on long-term use, selective Cyclooxygenase-2 inhibitors were also used. Only one COX-2 inhibitor in the prescriptions confirms the selective use of these drugs, due to cardiovascular risk.[9]

Erythrocyte sedimentation rate is the most frequently and commonly used laboratory parameter to assess the inflammation or disease activity in RA. Worsening disease is usually associated with an increase in ESR, and remission with normalization of ESR. In this study the DMARD combination with steroids was found to be effective. The multiple mechanisms of these drugs in reducing the inflammatory mediators could be the contributing factor. NSAIDs are considered to be poor anti-inflammatory drugs when compared to DMARDs and steroids.[10]

VAS measurements are simple and widely used as a tool to measure pain and fatigue. The present study revealed pain reduction, improvement in fatigue with combination of NSAIDs to regular treatment regimens.

Low-titers of RF value indicate good response to the treatment. Since the RF titers were not known, it could not be correlated to the clinical improvement.[11]

## CONCLUSION

The results of this study suggest that DMARD with steroid and NSAID combination might be effective for the treatment of rheumatoid arthritis. However, a larger study population and more appropriately powered sample would be required to set standard treatment regimens.

## ACKNOWLEDGEMENTS

We would like to thank Dr.Dinakar Rai and other practitioners of the orthopedic unit for their cooperation. We thank Mrs.T.Gayathri, Mr.K.G.Prasanth, Mrs.C.Kalaiyarasi & Mr.B.Balaji for their constant encouragement. Most importantly, we thank all the patients who participated in this study for their time and patience.

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# Formulation and Evaluation of Polyherbal Tablets for the Treatment of Leucorrhea

Formulation And Evaluation of Polyherbal Tablets For The Treatment of Leucorrhea
N.Deattu*[1], N.Narayanan[2], L.Suseela[3]
[1]College of Pharmacy, Madras Medical College, [2]Jaya College of Pharmacy, Chennai
[3]Jamia Salafia College of Pharmacy, Calicut
*For Correspondence e-mail: ndeattu@gmail.com

Received Date : 10.07.2012 Accepted Date : 20.07.2012

## ABSTRACT

Leucorrhea commonly known as 'whites' refers to a whitish discharge from the female genitals. It is an abnormal condition of the reproductive organs of women. In the present study an attempt has been made to develop a new polyherbal formulation for the treatment of leucorrhea. The polyherbal tablet formulation was developed from the combined ethanolic extracts of Saraca indica, Symplocos racemosa, Hemidesmus indicus, Aloe vera, Asteracantha longifolia, Erythrina indica and Tribulus terrestris. The polyherbal extract (PHE) was evaluated for in vitro antitrichomonal activity. The PHE was found to possess significant antitrichomonal activity. The PHE was compressed into tablets using various excipients, viz., micro crystalline cellulose, dicalcium phosphate, cross caramellose sodium, talc, magnesium stearate, and aerosil.

**Key words :** Polyherbal extract, In vitro antitrichomonal activity, Polyherbal tablets.

## INTRODUCTION

Leucorrhea commonly known as whites refers to a whitish discharge from the female genitals[1]. Leucorrhea is a very common complaint in the life of women. Commonly fungal, parasitic, bacterial and sexually transmitted diseases are the prime causative factors[2].

For many years, various indigenous preparations have had a reputation of efficacy in the treatment of this disorder. Nowadays, the synthetic drugs are although dominating the market, the element of toxicity of these drugs cannot be ruled out. As a result of the adverse reactions of synthetic and chemical medicines observed round the globe, herbal medicines have made a comeback to improve our basic health needs. With the emerging interest in the world to adopt and study the traditional system and to exploit their potentials based on different health care systems, the evaluation of the rich heritage of the traditional medicine is essential[3].

Globally there is resurgence in usage of plant based medicines and WHO also recommends the same because of its holistic approach with maximum therapeutic efficacy and minimum side effects it provides as compared to conventional medicine[4]. Herbal medicines can have as potent pharmacological effects as any conventional medicine[5]. It is believed that treatment with natural remedies has long lasting effects. An extensive literature survey was carried out to explore the various preparations employed in the treatment of Leucorrhea. It was evident from the literature survey that allopathic medicines are available and their side effects cannot be ruled out. It was also explored that some herbal products are also used for the treatment of this disease but these preparations are not subjected to standardized procedures. Hence an attempt has been made to develop a new polyherbal formulation for the treatment of Leucorrhea from a combination of plant extracts which is more effective. The developed formulation is prepared from the ethanolic extract of Saraca indica, Symplocos racemosa, Hemidesmus indicus, Aloe vera, Asteracantha longifolia, Erythrina indica and Tribulus terrestris.

**MATERIAL AND METHODS:** The extract of the plants was obtained from Rumi Herbals Research and Development, Chennai – 37. The excipients used for the formulation were obtained from MMC Health Care, Pvt. Ltd, Chennai - 124.

**Preparation of extract:**

The freshly collected plants were shade dried and powdered to coarse size. It was then extracted with various solvents in increasing order of polarity by Soxhlation. The ethanolic extract was used for the present study.

**Evaluation of in vitro antitrichomonal activity:6,7**

Antitrichomonal activity was studied with the polyherbal extract.

**Parasite isolate:**

Trichomonas vaginalis used in the experiment was taken from patients with pelvic inflammatory diseases or vaginal discharge complaints by pap smear technique and confirmed by wet mount preparation.

**CPLM ( Cystein-Peptone-Liver-Maltose ) cultivation method:**

The culture was done according to the technique described by Oyerinde with slight modification8. Pap smear samples from Leucorrhea were collected in CPLM medium. The cultivated materials were incubated at 37 ± 1.0°C in anaerobic condition and their microscopic examination were done after 24 and 48 h by taking a drop from the bottom of the culture using sterile Pasteur pipette, transferring to a slide and examined under high power objective.

**Inoculums:**

Trichomonas. vaginalis was inoculated in the RPMI ( Roswell Park Memorial Institute ) 1640 medium and incubated at 37 ± 1.0° C for 48 h. Parasites were counted under the microscope using haemocytometer chamber.

**In vitro susceptibility assays:**

5 mg of extract was dissolved in 50 µl of dimethylsulfoxide (DMSO) in eppendorf tube containing 950 µl of distilled water in order to reach concentration of 5 mg/ml (5000 ppm). The concentrates were stored at -20°C for further analysis. Sterile 96-well microtite plate was used for the plant extract, positive control and negative control. Each test included metronidazole pure compound (5 mg), a trichomonocide was used as positive control in concentration 312.5 mg/ml, whereas untreated cells used as negative control (culture medium plus trophozoites). Samples were taken for counting at 0, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h. For counting, the samples were mixed with trypan blue in equal volume. The final number of parasites was determined with haemocytometer in triplicate. The mortality % of parasite was carried out according to the following formula:

Mortality of parasite (%) = (Control negative – tested sample with extract) / Control negative x 100%

Only 100% inhibition of the parasite considered, when there was no motile parasite observed. The time duration in hours taken for mortality of Trichomonas vaginalis is given in Table No.1.

**Statistical analysis:**

All data are presented as means ± SD. Student t test was used to determine significant difference between control and polyherbal extract at level of $p < 0.05$.

**Preparation of polyherbal formulation:**

The plant extract was mixed with the excipients and compressed into tablets. The quantity of individual plant extract used for the formulation is shown in Table No.2.The quantity of extract used was in accordance with the literature collected and on the basis of the formulations available in the market. Ten trial batches F1, F2, F3, F4, F5, F6, F7, F8, F9 and F10 were prepared as shown in Table No.3.

**Procedure:**

The following sequence of processes were adopted to prepare the film coated polyherbal tablets of all ten formulations like weighing, granulation, drying, lubrication, compression, and coating9,10.

**Granulation:**

An accurately weighed quantity of the extract, microcrystalline cellulose and dicalcium phosphate passed through sieve no.20 and starch passed through sieve no.100 were transferred into a rapid mixer granulator and granulated with purified water and mixed for 15 minutes. The granules were transferred to a tray drier and the granules were dried at 40°C ± 5°C for 4 h. The dried granules were then passed through sieve no.14. The dried granules were transferred to a double cone blender and lubricated with talc, cross caramellose sodium, magnesium stearate and aerosil passed through sieve no.20. The lubricated granules were evaluated for following parameters like loss on drying, water content, particle size distribution, angle of repose, bulk and tapped density, Carr's index and Hausner's ratio.

**Compression and Coating:**

The lubricated granules were compressed by using punches of 17.5 x 7 mm'D' Tooling (Fluid Pack Acquira) Rotory tablet press and film coated. The tablets were evaluated for the parameters like uniformity of weight, friability, thickness, length and breadth, hardness and disintegration.

**Packaging:**

The coated tablets of F3 formulation were packed in blister pack (10×10's) using polyvinylidene chloride (PVDC) and aluminum foil as packing material.

**Selection of Suitable Formulation:**

The test reports of the various stages of the formulation process such as granulation, compression and coating of all the ten formulations were critically verified. The results for all the formulations were within the acceptable limits. However the results revealed that among the ten formulations the formulation F3 was found to have better compliance. The granules obtained for the trial batch F3 was found to be satisfactory. No rat holing was observed during the flow of granules from the hopper. Capping and sticking were not observed. Further the granules of batch F3 exhibited good flow properties

## RESULTS AND DISCUSSION

The present study was undertaken to develop a new polyherbal formulation for the treatment of Leucorrhea. The formulation was made from the ethanolic extract of a combination of plants, viz., Aloe vera, Asteracantha longifolia, Erythrina indica, Hemidesmus indicus, Saraca indica, Symplocos racemosa and Tribulus terrestris. The PHE was subjected to in vitro antitrichomonal activity and it was found to possess significant antitrichomonal activity The extract of plants was mixed with the excipients and compressed into tablets. Ten trial batches F1, F2, F3, F4, F5, F6, F7, F8, F9 and F10 were prepared. The trial batch F3 was found to be satisfactory. The tablets were evaluated for hardness, thickness, uniformity of weight, friability and disintegration time. The maximum weight variation of the tablets was ± 0.8% which is within the acceptable weight variation range of ± 5%. Hardness was found to be 6.2 kg/cm$^2$ which falls above the limit of not less than 3.0 kg/cm$^2$. Friability value was 0.5%W/w which is not more than the acceptable value of 1.0% w/w. The thickness of the tablets was found to be 4.7mm indicating fairly acceptable values. An ideal tablet should disintegrate within 15 min. The tablets of batch F3 disintegrated within 3 minutes. Thus the results of tablet evaluation proved that all values were within the acceptable limits.

## CONCLUSION

In the present research work an attempt has been made to develop a new polyherbal formulation for the treatment of Leucorrhea from the extract of various plants viz, Aloe vera, Asteracantha longifolia, Erythrina indica, Hemidesmus indicus, Saraca indica, Symplocos racemosa and Tribulus terrestris. The PHE showed good antitrichomonal activity. The PHE was granulated with excipients, the formed granules were compressed and film coated. Better therapeutic effect and good patient compliance are the reasons for choosing drug from natural origin. Based on the above facts a new polyherbal formulation has been successfully developed for the treatment of leucorrhoea.

## ACKNOWLEDGEMENTS

We are thankful to Rumi Herbals Research and Development, Chennai – 37 and MMC Health Care, Pvt. Ltd, Chennai – 124 for providing us the necessary materials required for our research work.

**Table 1 : Patients Characteristics and Disposition**

| Group | Time / Hours | | | | | | |
|---|---|---|---|---|---|---|---|
| | 96 | 120 | 144 | 168 | 192 | 216 | 240 |
| Positive control | 76 | 78.5 | 80.5 | 84 | 88 | 92.5 | 98.5 |
| Negative control | 0 | 7 | 14 | 20 | 30 | 35.5 | 40 |
| 250 ppm | 70 | 80 | 82.5 | 84.5 | 88 | 89.5 | 89.5 |
| 500 ppm | 80 | 84.5 | 89.5 | 91.5 | 92 | 94.5 | 95.5 |
| 750 ppm | 83.5 | 89.5 | 93.5 | 98.5 | 100 | 100 | 100 |
| 1000 ppm | 82 | 91.5 | 98.5 | 100 | 100 | 100 | 100 |

**Table No 2 : Quantity of plant extract used for preparing polyherbal formulation**

| S.No | Plant used | Quantity of extract (Each tablet contains) |
|---|---|---|
| 1 | Saraca indica | 10 mg |
| 2 | Symplocos racemosa | 10 mg |
| 3 | Hemidesmus indicus | 10 mg |
| 4 | Aloe vera | 5 mg |
| 5 | Asteracantha longifolia | 10 mg |
| 6 | Erythrina indica | 2.5mg |
| 7 | Tribulus terrestris | 2.5mg |

**Table No 3 : Formulation of tablets**

| Ingredients | Formulation | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | F1 | F2 | F3 | F4 | F5 | F6 | F7 | F8 | F9 | F10 |
| Extract | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| Microcrystalline cellulose | 250 | 300 | 352 | 400 | 452 | 0 | 202 | 152 | 100 | 52 |
| Dicalcium phosphate | 202 | 152 | 100 | 52 | 0 | 452 | 250 | 300 | 352 | 400 |
| Starch | 26 | 26 | 26 | 26 | 26 | 26 | 26 | 26 | 26 | 26 |
| Cross caramellose sodium | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 |
| Talc | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 |
| Magnesium stearate | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 |
| Aerosil | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| Purified water | QS | QS | Q S | QS | QS | QS | QS | QS | QS | QS |

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# Study on *In Vitro* Antioxidant Activities of Methanolic Leaf Extract of Swietenia Mahagoni

S.Vanitha*[1], T.Venkatarathinakumar[2], Ajithadas Aruna[2], R.Gowri[2],

[1]P.S.G College of Pharmacy, Department of Pharmacognosy, Coimbatore-641004.

[2]Department of Pharmacognosy, Madurai Medical College, Madurai-625020.

*Corresponding Author Email: vanithavel2002@yahoo.in

Received Date : 11.04.2012 Accepted Date : 30.06.2012

## ABSTRACT

Antioxidant potential of Swietenia mahagoni leaf extract was analyzed for its antioxidant (1,1-diphenyl-2-picryl hydrazyl and ferric reducing power methods) and phytochemical analysis. The extract was found effective against the antioxidant test models exhibiting an IC 50 value 21.22957µg/ml and for Vitamin-c 17.96255µg/ml (% inhibition for the extract was found to be 95.87 ± 0.89% and for standard was 96.94 ± 0.043%). FRAP assay revealed that 50 µg/ml of this extract showed the absorbance of 0.564, where as the absorbance of standard drug was 0.598. The results indicate that IC50 value of the methanolic extract was found to be low and an increase in absorbance in FRAP assay, that showed strong potential antioxidant activity in both assays.

**Key words :** Antioxidant, DPPH assay, Ferric reducing power assay, Swietenia mahagoni leaves

Swietenia mahagoni (L) jacq (Meliaceae) is a large, evergreen, deciduous and economically important timber tree native to the West Indies. This tree is mainly cultivated at tropical zones, such as India, Malaysia and southern china1. This plant is used in African traditional medicine as a popular remedy to treat various ailments. The ethanomedical information revealed that decoction of the bark of these mahagonies is extensively used as febrifuge, which could be associated with it's used as antimalarial drug. Swietenia mahagoni seeds have been used as folk medicine for the treatment of hypertension, diabetes, and malaria[2], and they have also been reported to have medicinal value for treatment of cancer, amoebiasis, coughs, chest pains and intestinal parasitism. Leaf decoction of this plant is used to treat nervous disorders and also to cure malaria, anemia, diarrhoea, fever, dysentery and depurative[3]. The leaves contains several limonoids; seven phragmalin limonoids of swietephragmins A-G as well two other different types of 2-hydroxy-3-O-tigloyl-swietenolide and deacetylsecomahoganin4 which are responsible for these therapeutic effects[4]. The present investigation was undertaken to evaluate the antioxidant activity of leaf extracts of Swietenia mahagoni.

The leaves of Swietenia mahagoni (L) jacq are collected in Alagarkovil Temple, Madurai dist, Tamilnadu state, India. The plant was identified and authenticated by Senior Plant Taxonomist - Plant Anatomy Research Centre (PARC/2011/787) at Chennai-5. The shade dried leaves were powdered and defatted with petroleum ether. Then it was extracted with methanol (A.R grade) by cold maceration. Preliminary phytochemical screening was carried out [5,6,7] and presented in [Table 1]. Antioxidant activity of the plant extracts was studied by 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) assay and ferric-reducing power assay models. In vitro DPPH radical scavenging activity was carried out by adopting the method of Blois [8].

The DPPH solution (0.1mM) in ethanol was prepared and 1.0ml of this solution was added to 3.0 ml of extract from 50µg/ml concentration and for standard at different concentrations (10-50 µg/ml) in water. Thirty minutes later, the absorbance was measured at 517 nm using UV/Vis spectrophotometer. Scavenging of DPPH radical was calculated using the following equation.

$$\% \text{ inhibition} = A0\text{-}A1/A0 \times 100$$

Where

A0-the absorbance of the control reaction

A1- the absorbance of the test sample

IC 50 value was also calculated, ascorbic acid was used as the reference standard.

The reducing power of the ethanol extract was carried out by adapting the method of Oyaizu9 FRAP reagent was prepared by mixing 25 ml of acetate buffer (500 mM/l) with 2.5 ml of tripyridyltriazine (TPTZ) (10 mM/l) and 2.5ml of ferric chloride (20 mM/l) solution. The reaction mixture contained 300ml of freshly prepared FRAP reagent warmed to 37oC, added to 10ml various conc. (10-50µg/ml) of test along with 30ml of water. Absorbance of this solution was taken at 593 nm, just after 4 min from the time of addition of FRAP reagent. An increase in absorbance indicated enhanced reducing potential of methanolic extract. The standard curve was plotted using ferrous sulphate solution at different conc.10-50µg/ml.

The result of phytochemical analysis was recorded in [Table 1]. The leaf extract of Swietenia mahagoni (L) jacq exhibited potential scavenging antioxidant activity. % inhibition for the extract was found to be 95.87 ± 0.89% and for standard was 96.94 ± 0.043%. IC50 values of MESM leaf was 21.23µg/ml. It was compared with IC50 values of standard reference drug (Ascorbic acid) was 17.96µg/ml. 10 µg/ml of this extract showed absorbance of 0.212 ± 0.017 where as 0.235 ±0.001 for standard drug FeSO4. Methanolic extract at 50µg/ml showed the absorbance of 0.564 ± 0.03, where as the absorbance of standard drug was 0.598 ± 0.001. FRAP assay revealed that an increase in absorbance indicated enhance reducing potential of sample. The leaf extract of Swietenia mahagoni (L) jacq exhibited potential antioxidant activity in the both the assay models.

**Table 1: Phytochemical Analysis of Swietenia Mahagoni(L) Jacq**

| S.NO | Chemical components | Leaf extracts |
|---|---|---|
| 1. | Alkaloids | - |
| 2 | Saponin | + |
| 3 | Tannin and phenolic compounds | + |
| 4 | Flavonoids | + |
| 5 | Steroids | + |
| 6 | Oils and fats | - |
| 7 | Terpenoids | + |
| 8 | mucilage | - |

**(+) indicates positive reaction (-) indicates negative reaction**

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|---|---|---|
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| Mass | gram | g |
| | kilogram | kg |
| | microgram | pg |
| Time | second | s |
| | minute | min |
| | hour | h |
| | day | d |
| | week | w |
| | month | mo |
| | year | y |
| Amount of substance | mole | mol |
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| Volume | cubic meter | $m^3$ |
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| | milliliter | ml |
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| When numbers are printed symbols are preferred | 100 meters | 100 m |
| Space between number and symbol | 2mol | 2 mol |
| Place a zero before a decimal | 10mg | 10 mg |
| Decimal numbers are preferable to fractions | 0.75 | |
| Space used to separate long number exception four digit numbers | | 15000001000 |

**Spelling:-** There should be as in the Concise Oxford Dictionary of Current English.

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**V. Sankar. Ampicillin prodrugs:** amide conjugates from aminoacids and ampicillin, pharmazle 2001, 56(7), 588.

**Books and other monographs:-** Personal author(s): C. Vijaya Raghavan and Judith Justin. Experimental Biopharmaceutics and Pharmacokinetics. 1st Edn. India: New Century Book House Publishers: 2006.

**Editor(s), Compiler(s) as author:-** Norman IJ, Redfern SJ, editors. Mental health care for elderly people. New York: Churchill Livingstone; 1996.

**Chapter in a book:-** Philips SJ, Whistant JP. Hyertentsion and stroke. In: Laragh JH, Brenner BM, editors. Hypertension: pathophysiology, diagnosis, and management. 2nd ed. New York: Raven Press; 1995.p.465-78.

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**Patent:-** Nilani. P, Sankar. V, Chandrasekharan. A. K. Herbal Ant Repellant. INDIA patent: 2007-09-14, filed 2004-09-29.

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**Available from URL:-** http://ccic.gov/nddod/EID/eld.htm

**World Wide Web Format:-** Author/editor (surname initials). Title [online]. Year [cited year month day].

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