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Research Article

COMPARATIVE GC-MS ANALYSIS AND IN VITRO SCREENING OF FOUR SPECIES OF MUCUNA

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ABSTRACT

The herbal market is flooded with substituent and adulterants due to unavailability of the correct drug and less knowledge on the identity of the drugs. *Mucuna pruriens* is used as the medicament from herbal source to treat the Parkinsonism. *Mucuna pruriens* and other species of *Mucuna* were subjected to GC-MS analysis to find out the difference in their phyto-constituents. The comparative *in vitro* studies for reducing ability, inhibition of denaturation, anthelmintic activity and membrane stabilizing activities of *Mucuna* species were carried out to find out the efficacy profile for antioxidant, anti-inflammatory and anthelmintic activities. The other species of *Mucuna* are also having the same activities of *Mucuna pruriens* with a difference in percentage. Results of GC-MS shows the common ingredients present in all varieties with the slight difference in the fragments.

Keywords: *Mucuna*, Gas Chromatography, *In vitro* study, adulterants, Parkinsonism, Reducing ability

INTRODUCTION

Mucuna pruriens (L.) DC., the velvet bean, is one of the important herbal drugs in Siddha, Ayurveda and Unani systems of medicine. Seeds of this plant are rich in L-DOPA content. They have been used both as food and medicine for many other common diseases. The seeds have been sold in the herbal drug stores in many parts of India as 'Atmagupta' or 'Poonaikali'. In our preliminary market survey we found that seeds of many other species other than *M. pruriens* are sold as Poonaikali' in Tamil Nadu and 'Atmagupta' or 'Kawanch' in other states of India. It is alarming to note that the crude drug traders and traditional physicians and pharmaceutical manufactures, which use this seed for preparation of medicine, are unaware of its identity and its adulterants. Being a common drug and great demand in India and abroad, it is essential that a standard is to be established on scientific lines for identifying the authentic drug and to detect its adulterants. Though many pharmacological works on seeds of *M. pruriens* were documented scientifically, but no research work on comparative study of *M. pruriens* and its adulterants is available[1].

The seeds of *Mucuna pruriens* are said to have Antiparkinson activity along with other activities. Many times *Mucuna pruriens* (MP) has been adulterated with other species of the genus like *Mucuna cochinchinesis* (MC), *Mucuna utilis* (MU), *Mucuna deeringiana* (MD). Previously detailed pharmacognostical work has been carried out in *Mucuna* seeds and its adulterants along with the basic phytochemical and pharmacological works[2]. In the present work, comparative GC-MS and *invitro* studies were performed in *Mucuna pruriens* and its adulterants to complete the gap in the previous work. Common adulterants of *Mucuna pruriens* are *Mucuna cochinchinesis, Mucuna utilis and Mucuna deeringiana.*

MATERIAL AND METHODS

Collection of Seed Samples

Seeds of 'poonaikali' (2 kg) were purchased from different drug stores in Madurai, Thanjavur, and Chennai. Some of the seed samples were also grown in Tamil University Herbal Garden, Thanjavur District, Tamilnadu, India. The collected seeds were authenticated by Dr. M. Jegadeesan, Department of Environmental Sciences and Medicinal Botany, Tamil University, Thanjavur, Tamilnadu, India. A voucher specimen has been deposited in the Herbarium of Department of Environmental Sciences and Medicinal Botany, Tamil University, Thanjavur District, Tamilnadu state, India.

Preparation of Extract

The collected seed samples were thoroughly dried in the open sunlight for 2 days. Then the dried seeds were cleaned and any foreign matter, broken seeds and immature seeds were removed. The seeds were stored in a plastic container at room temperature. Then the seeds were powdered separately in a mechanical way to 60 mesh size. The seed powder was soaked in 70% ethanol for 72 hours with occasional shaking. The solvent was decanted and filtered. The marc was subjected to further extraction by repeating the procedure thrice. The solvent was removed from the extract by vacuum distillation.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The four different Mucuna extracts were dissolved in methanol and analyzed by GC–MS on GC Clarus 500 Perkin Elmer using the following experimental conditions: Column type - Elite -5 (5 % diphenyl 95 % dimethyl polysiloxane), Column dimension 30 m X 0.32 mm), carrier gas – Helium 1 ml/min, column temperature from 50°C up to 285°C at the rate of 10 °C/min and 5 min hold, at 285 °C, injector and detector temperature - 290°C, injection mode split, volume injected: 0.5 µl of a solution prepared from 2 mg/100 ml in methanol. Total run time was 30 minutes. Mass spectrum was taken using Mass detector – Turbo Mass gold – Perkin Elmer. Transfer line temperature – 230°C, Source temperature – 230°C, scan range is from 40 – 450 amu, ionisation technique – Electron ionization technique.

Detection of the compounds

Diluted samples (1/100, v/v in methanol) of 1.0 µl were injected manually in the split less mode. The relative percentage amount of each component of four extracts of *Mucuna* species was calculated by comparing its average peak area to the total areas.

In-vitro studies

Anthelmintic Bioassay

The earthworm *Pheretima posthuma* (Annelida, Megascolecidae) was used for evaluating the anthelmintic activity of crude extract using the reference substance for comparison. Earthworms were procured from Periyar Maniammai University, Thanjavur, Tamilnadu and maintained at Centre for Advanced Research in Indian System of Medicine, SASTRA University, Thanjavur, Tamilnadu.

Anthelmintic activity was assessed using earthworms by the reported methods with slight modification[3]. The assay was performed using adult Indian earth worm, *Pheretima posthuma* due to its anatomical and physiological resemblance with the intestinal roundworm parasite of human beings[4,5]. Because of easy availability, earthworms have been used widely for the evaluation of anthelmintic compounds *in vitro*[6,7]. Piperazine citrate at the dose of 10 mg/ml dissolved in distilled water was used as reference. The hydrolacholic extract of *Mucuna cochinchinensis* (MC), *Mucuna deeringiana* (MD), *Mucuna pruriens* (MP) and *Mucuna utilis* (MU) were dissolved in distilled water and at the dose of 100 mg/ml was used for anthelmintic study. The extract and standard (25 ml) was poured into petri dish and six worms of about the same size per petridish were used. The worms were observed for their spontaneous motility and evoked responses. Time for paralysis was noted when no movement could be observed except when the worms were shaken vigorously. Time for death of worms were recorded after ascertaining that worms neither moved when shaken vigorously nor when dipped in warm water (50°C), which stimulated and induced movements if the worm was live and followed by fading away of their body colours.

Membrane stabilizing activity

Membrane stabilizing activity was evaluated by the method described by Shinde *et al* (1999) with some modifications[8]. Whole human blood was obtained from a healthy human volunteer and transferred to heparinized centrifuge tube. The blood was washed three times with isotonic buffered solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4) and centrifuged for 10 minutes at 3000g. The test sample consisted of stock erythrocyte (RBC) suspension (0.5 ml) was mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the hydroalcoholic extract of *Mucuna* species (100 µg/ml) or Acetyl salicylic acid (100 µg/ml). The control sample consisted of 0.5 ml of RBC suspension mixed with hypotonic buffered saline solution alone. The mixtures were incubated for 10 min at room temperature and centrifuged for 10 min at 3000g and the absorbance of the supernatant was measured at 540 nm. Each experiment was carried out in triplicate and the average was calculated. The percentage inhibition of haemolysis or membrane stabilization was calculated by following equation:

% Inhibition of haemolysis = 100 x (A_1-A_2/A_1)
Where:
A_1 = Absorption of hypotonic buffered saline solution alone

A_2 = Absorption of test sample in hypotonic solution

Effect on protein denaturation

Test solution (1ml) containing of hydroalcoholic extract of four different *Mucuna* species (100 µg/ml) or Acetyl salicylic acid (100 µg/ml) was mixed with 1ml of egg albumin solution (1mM) and incubated at 27 ±1 °C for 15 min. Denaturation was induced by keeping the reaction mixture at 70 °C in a water bath for 10 min. After cooling, the turbidity was measured spectrophotometrically at 660 nm[9,10]. Percentage inhibition of denaturation was calculated from control where no drug was added. Each experiment was carried out in triplicate and the average was taken.

Reducing ability

Reducing power of the test samples was determined on the basis of the ability of their antioxidant principles to form colored complex with potassium ferricyanide, trichloroacetic acid (TCA) and Ferric chloride[11]. The extract (1 ml) of different concentrations (25, 50, 100, 200, 400 µg/ml) were mixed with potassium ferricyanide (2.5 ml, 1%) and 2.5 ml of phosphate buffer (pH 6.6). The mixture was incubated at 50°C for 20 minutes and TCA (10%, 2.5 ml) was added to it. The mixture was centrifuged at 3000 rpm for 10 min. the supernatant (2.5 ml) was pipette out and mixed with 2.5 ml water and 0.5 ml $FeCl_3$ (0.1%). Absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power.

RESULTS

The GC-MS analysis of *Mucuna cochinchinensis* reveals the presence of 49 compounds. Pyrolidine derivatives, galactopyranoside derivatives, glucopyranoside derivative and cinnamic acid derivatives are important among them. The chromatogram of *Mucuna deeringiana* shows the presence of thirty six phytoconstituents in total. Arbutin, adenine, palmidrol and glucopyranoside derivatives are important of then. GC-MS analysis of *Mucuna pruriens* indicates the presence of fourty three compounds in total. Tocophonol, Ricinoleic acid, glucopyranoside derivatives and aziridine derivatives are important phytoconstituents noted. *Mucuna utilis* is having more quantity of higher fatty acids like Hexadecanoic acid esters and Octadecanoic acid esters. (Fig 1 and Table 1)

The hydroalcoholic extract of all the *Mucuna* seeds showed no significant paralytic activity compared to standard drug. Among the four seed extracts, *Mucuna deeringiana* showed significant anthelmintic activity comparable to the standard drug. Whereas other three seed extracts did not show any significant anthelmintic activity. (Table 2)

Acetylsalicylic acid (100 µg/ml) offered a significant ($p < 0.05$) protection against damaging effect of hypotonic solution. At the concentration of 100 µg/ml, hydroalcoholic extract of MC, MD, MP and MU showed 28.57, 10.00, 14.28 and 14.28 % respectively; whereas acetylsalicylic acid showed 67.14% inhibition of RBC heamolysis when compared with control (Table 3). All the hydoalcoholic extracts at 100µg/ml concentration did not significantly inhibit haemolysis. *Mucuna cochinchinesis*, however showed maximum inhibition.

The inhibitory effect of hydroalcoholic extract of MC, MD, MP and MU on heat induced protein denaturation is tabulated (Table 3). Among the four seed extracts of *Mucuna* species, MC showed maximum protein denaturation (68.75%) followed by MU. MP extract had minimum denaturation (36.66%) whereas acetylsalicylic acid showed 76.64 % inhibition of protein denaturation.

Table 1: Comparative GC-MS analysis of compounds identified by four species of mucuna seed extracts

S. No.	Peak Name	% Peak area			
		MC	MD	MP	MU
1	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	0.62	-	2.02	1.02
2	3-Acetylthymine	1.86	1.11	1.80	-
3	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	1.36	0.74	1.73	1.48
4	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	0.04	0.02	0.09	0.08
5	1,2-Benzenediol, 4-methyl-	0.12	0.05	0.23	0.10
6	Sucrose	6.30	-	5.60	6.63
7	Ethyl à-d-glucopyranoside	59.06	32.59	53.17	47.38
8	3-O-Methyl-d-glucose	1.30	2.46	0.58	0.85
9	p-Arbutin	0.18	1.10	-	0.37
10	Hexadecanoic acid, methyl ester	1.98	-	2.71	16.12
11	n-Hexadecanoic acid	2.33	1.11	3.69	-
12	Hexadecanoic acid, ethyl ester	3.08	8.29	2.90	1.91
13	9,12-Octadecadienoic acid, methyl ester,	3.86	0.07	7.68	3.54
14	Palmidrol	1.32	1.70	-	2.13

15	9,12-Octadecadienoic acid, ethyl ester	3.95	2.82	5.32	2.16
16	Decanamide, N-(2-hydroxyethyl)-	0.22	0.17	3.02	2.07
17	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	2.38	1.67	-	1.54
18	Vitamin E	-	-	0.56	-

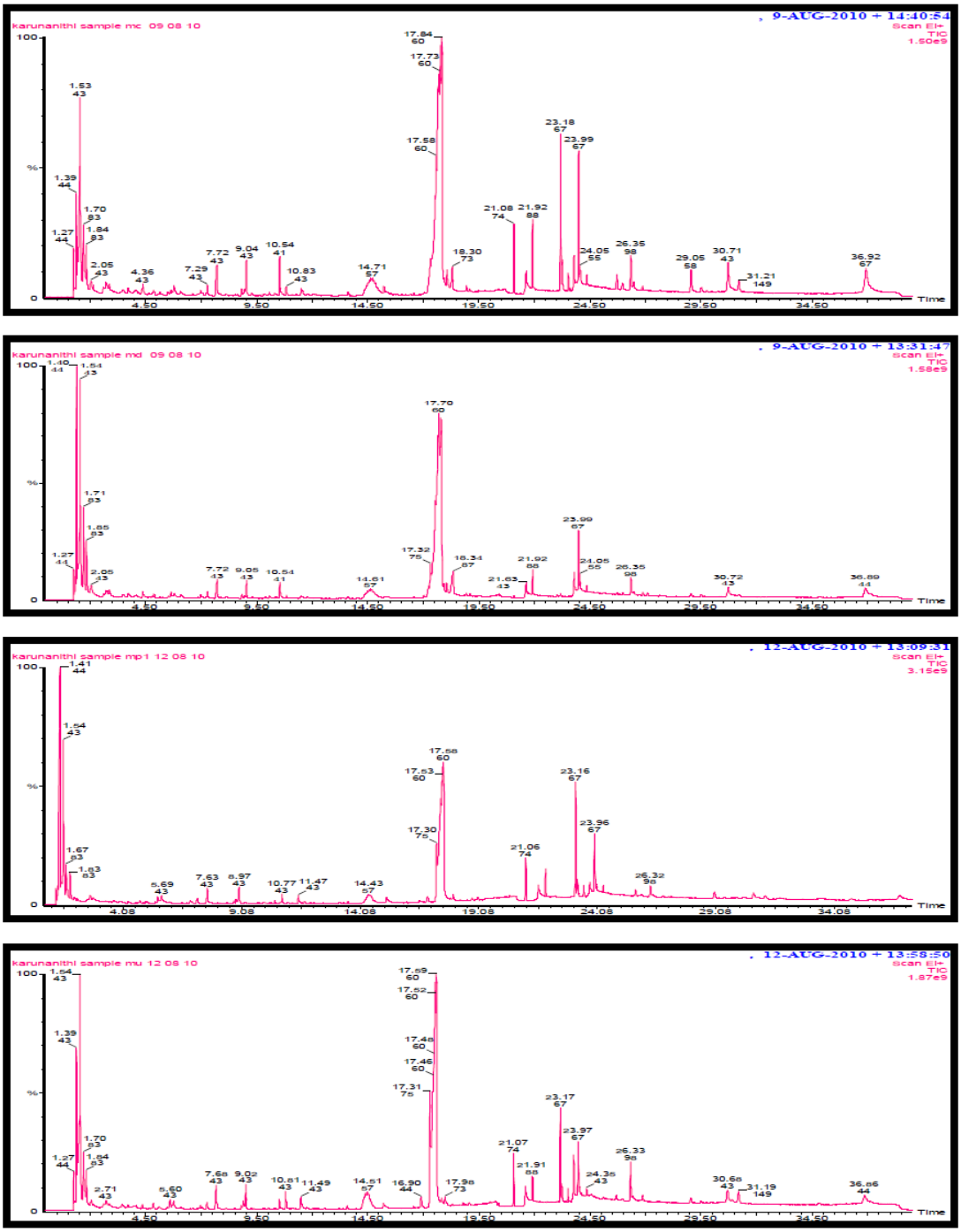

Fig 1: GC-MS chromatogram of MC, MD, MP and MU extracts.

Table 2:Anthelmintic activity of hydroalcoholic extract of MC, MD, MP and MU

Treatments	Concentration (mg/ml)	Time for Paralysis (Min)	Death (Min)
Piperazine citrate	10	8.00±0.89	473.80±13.87
Mucuna cochinchinesis	100	315.30±8.74**	>1440.00±0.00**
Mucuna deeringiana	100	219.70±7.27**	435.70±16.27
Mucuna pruriens	100	209.50±25.44**	>1440.00±0.00**
Mucuna utilis	100	166.30±11.16**	810.20±17.04**

The values are in Mean ± SEM, n=6, **p<0.005 and compared with standard control.

Table 3: Effect of hydroalcoholic extract of MC, MD, MP and MU on hypotonic solution induced RBC haemolysis and heat induced protein denaturation

Treatments	Concentration	% Inhibition of Haemolysis	% inhibition of protein denaturation
Control	50 mM	-	-
Acetylsalicylic acid	100 µg/ml	67.14*	76.64*
Mucuna cochinchinesis	100 µg/ml	28.57	68.75*
Mucuna deeringiana	100 µg/ml	10.00	44.11
Mucuna pruriens	100 µg/ml	14.28	36.66
Mucuna utilis	100 µg/ml	14.28	48.48

The values are in Mean ± SEM, n=3, *$p < 0.05$ compared with control group.

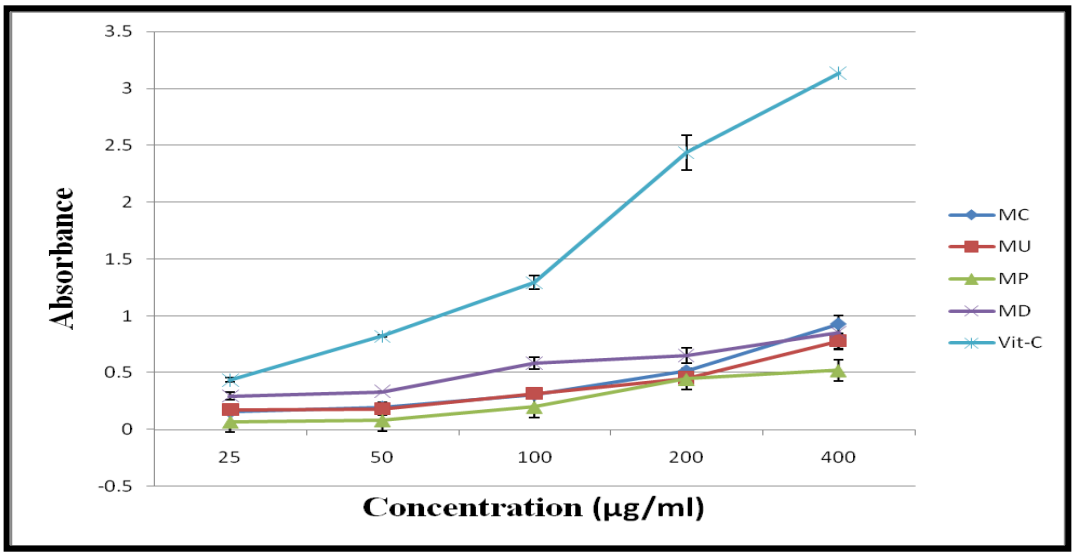

Fig 2: Reducing power of hydroalcoholic extract of MC, MD, MP and MU extracts.

The reducing power of hydroalcoholic extract of MC, MD, MP and MU as a function of their concentration was evaluated and shown in Fig 2. In this assay, the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers (i.e. antioxidants) causes the conversion of the Fe^{3+}/ferricyanide complex used in this method to the ferrous form. Therefore, by measuring the formation of Perl's Prussian blue at 700 nm, we can monitor the Fe^{2+} concentration; a higher absorbance at 700 nm indicates a higher reducing power. The reducing power of hydroalcoholic extract of MC, MD, MP and MU increased with concentration. Reducing powers obtained for all the extracts at the concentration of 100 µg/ml were found in the order: MD>MU>MC>MP. Reducing powers of Vitamin C at 100 µg/ml was 1.29. It was reported that the reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom[12].

DISCUSSION

The GC-MS analysis of hydroalcoholic extracts of MC, MD, MP and MU were giving total number of peaks of 49, 37, 42 and 43 respectively. Seed samples of *Mucuna* contain important compounds like glycopyranoside, O-D- Methyl- glucose, Hexadecanoic acid and its esters, Octadecanoic acid and its esters, Arbutin, palmidrol and acetyl thymine. Glycopyranoside is an antioxidant, enhances intestinal calcium transport and bone calcium mobilization and a neuroprotectant[13,14]. O-D- Methyl– glucose found in all *Mucuna* spp. prevents brain lactate rise and has beneficial effects in minimizing the neuropathological consequences of ischaemic damage[15]. The higher fatty acid Hexadecanoic acid esters and Octadecanoic acid esters are well known antioxidant and neuroprotectant[16]. The presence of these antioxidant and neuroprotective compounds may be complementary and synergistic to the L- DOPA in the Parkinsonism treatment. The percentage peak area for hexadecanoic acid and octadecanoic and their esters are highest in *M. pruriens* and second highest in *M. utilis* and anti-parkinson activity is correspondingly more in these two species. Vitamin E is present in the GC-MS analysis of *M. pruriens* extract and absent in other three species of *Mucuna* under investigation. Vitamin E is a superior antioxidant and useful in nerve protection[17]. This tocopherol may be supporting the action of L- DOPA against Parkinsonism. Arbutin, a glycolated hydroquinone present in all the four seed samples is also having antioxidant activity[18].

In Anthelmintic assay, hydroalcoholic extract of MU at the dose of 100 mg/kg produced paralysis in 166.3 minutes and the paralysis time is very less, when compared with other three extracts of *Mucuna* species, whereas the standard piperizine citrate at the dose of 10 µg/ml produced only 8 minutes to paralysis and this is supported by its mechanism of action. The predominant effect of piperazine citrate on worm is to cause flaccid paralysis that result in expulsion of the worm by peristalsis. Piperazine citrate by increasing chloride ion conductance of worm muscle membrane produces hyperpolarisation and reduced excitability that leads to muscle relaxation and flaccid paralysis[19]. From the above results, it is evident that paralysis time for all the four extracts of *Mucuna* species is high when compared with the standard piperizine citrate and thus concluded that the four extracts doesnot have sufficient paralysis action on helminthes.

Protective effect on hypotonic saline-induced erythrocyte lysis is known to be a very good index of anti-inflammatory activity of any agent. Since the RBC membrane is similar to that of lysosomal membrane, inhibition of RBC heamolysis will therefore, provide good approaches into the inflammatory process especially as both events are also consequent of injury. Injury to lysosome membrane usually triggers the release of phospholipase A2 that mediates the hydrolysis of phospholipids to produce inflammatory mediators[20,21]. Stabilization of the membranes of these cells inhibits lysis and subsequent release of the cytoplasmic contents which in turn confines the tissue damage and exacerbation of the inflammatory response[22]. It is therefore expected that compounds with membrane stabilization activity should offer significant protection of cell

membrane against the release of injurious substances. *In vitro* assessment of acetylsalicylic acid at the concentration of 100 µg/ml on membrane stabilization showed inhibition on hypotonic solution induced haemolysis of red blood cells. *Mucuna pruriens* has ricinoleic acid, hexadecanoic acid and octadecanoic and their esters in GC-MS analysis of our study and this compound is having anti-inflammatory activity[23,24]. This ricinoleic acid may be responsible for this membrane stabilizing effect.

Denaturation of proteins is well documented and is caused by inflammation process, mostly in conditions like arthritis. Thus, the protection against protein denaturation which was the main mechanism of action of NSAIDs as postulated by Mizushima (1964) before the discovery of their inhibitory effect on cyclooxygenase, may play an important role in the antirheumatic activity of NSAIDs[25,26]. Production of auto-antigens in certain rheumatic diseases may be due to *in vivo* denaturation of proteins. The mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding[27]. The ability of hydroalcoholic extract of MC, MD, MP and MU to inhibit protein denaturation may contribute to its anti-inflammatory properties. Compounds like ricinoleic acid, hexadecanoic acid and octadecanoic and their esters bearing anti-inflammatory activity would be responsible for this protein denaturation inhibition[24,28].

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