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Research Article

Snake venom neutralizing effect of validated Herbal Medicine Formula Practiced in Tribal Medicine System (TMS) at B. R. Hills region of Karnataka, India

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ABSTRACT

The crude extract of Tribal Medicine Formulation (TMF), an herbal drug was taken to appraise the pharmacognostic status through antioxidant activity and anti -snake venom properties. The antioxidant activity has been evaluated by ABTS, TBARS and Hydroxyl radical scavenging assay methods, respectively. Since, ethno-medicinal plants and their formulation contain generally diverse class of active metabolites and phenolic compounds which possess wide-ranging amount of antioxidant activities; the anti -venom activity was evaluated for the enzymes like PLA_2 and protease which are present in the snake venom by interaction of proteins between *Naja naja* and protein of TMF drug. The Anti-PLA_2 activity was done to facilitate neutralizing effect of PLA_2 in association with the different fractions of TMF drug. Accordingly, the efficacy of TMF drug was employed to neutralize snake venom and the significant neutralization effect was noticed, which may be due to presence of certain active class of chemical compounds in TMF drug that acquired anti -lethal effects. Among them, presence of terpenoids, flavonoids, polyphenols, total sugars and ascorbic acid have contributed significantly as anti -venom potential. Therefore, multifunctionality of TMF drug has clearly indicated the competence of such active chemical constituents that make possible of binding between two proteins. Consequently, the obstruction in the functions of many macromolecules could be accomplished due to interaction of these two proteins. Later, the hemorrhage activity was conducted to assess the anti-snake venom activity through myonecrosis-lung hemorrhage activity. In the study, the mouse lung tissue and also the muscle tissue layer was collected and added with saline buffer to one layer in which, the cell death did not occur. Then, the other layer added with snake venom was found to be the cells started dying by forming tumors; whereas, the third layer which was added with both snake venom along with the extracts of TMF drug diminishes the activity of inflammatory enzyme. This confirms the better activity of TMF drug over venom which further influences neutralization of snake venom. However, it can be suggested that, the active metabolites present in the TMF drug extract are most responsible in inhibiting the PLA_2 activity and the fortification in the efficiency of drug was justified. This will facilitate the rationale factor pharmacologically which admits the traditional evidences for these Ethno-medicinal plants in the form of TMF drug as antidote for snake victims. Therefore, the TMF drug was found to have high potential active constituents which make possible of neutralization competence in treating snake bite. Further, the TMF drug can be explored for its therapeutic authentication through analyzing probable mechanism of action by lead molecules present in the formulations via clinical studies which can be recommended further as counteractive doctrine in case of snakebite envenomation.

Keywords: Tribal Medicine Formulation (TMF), Antioxidant activity, *Naja naja*, Anti Snake venom activity, Neutralization of Snake Venom, Anti-PLA_2 activity.

INTRODUCTION

The venomous snake bite' is a medicinal emergency needs immediate attention and the most bites of venomous snakes occur in the fields exclusively to the farming and as well as field labours. Therefore, the agriculture workers immediately look for antidote in the surrounding environment (Warrel, 1996; WHO, 2008 and Mohapatra *et al*, 2011). Most often, the

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available anti-dote depends on the effect of known plants comprising of different parts such as leaves, stem, roots, tubers and seeds etc. as antidote (Agoro, 1978; Hati *et al*, 1992; Biondo *et al*, 2003; Ambikapathi *et al*, 2009; Paula *et al*, 2010 and Amui *et al*, 2011). In rural area, several medicinal plants are most commonly being used by traditional healers as a source valid medicine for venomous snake bite such as *Datura metel*, *Rubia chordifolia, Embelia ribes, Glyceria glabra* etc (Houghton and Osibogun, 1993; Soars *et al*, 2004; Marcussi *et al*, 2007). The high mortality rate due to bite of poisonous snakes namely, Indian cobra, Saw scaled viper, Russell's viper, Common krait was noticed in the country are, etc. The study most frequently conducted was on Indian Cobra (*Naja naja*) or Spectacled Cobra, which is a species of the genus *Naja* found in the Indian subcontinent. The *Naja naja* is a member of the "big four", the four species which inflict the most snakebites in India (Barik, 1999; Asuzu and Harvey, 2003; Alam and Gomer, 2003; Ashok, 2004; Acharya *et al*., 2008 and Ambikabothya *et al*, 2011).

About Snake Venom

The venom derived from snakes comprises of multifarious factors that, the effectiveness is varied with respect different species that can hold a variety of toxins. The components of toxin can principally include Phospholipase A_2 as predominant factor followed by proteases, nucleases, phospho-diesterases and other enzymes which disrupt physiological processes and cellular integrity in the biological system. In addition, the major classifications of venom toxins include largely are neurotoxins, cytotoxins, myotoxins and cardiotoxins respectively. The venom will be produced in the modified parotid glands of snake that is responsible for secreting saliva. The venom is stored in alveoli which is a specialized structure located just behind the eyes of the snake and the venom will be ejected vol untarily from its hollow tubular fangs. Apart from other class of substances, the snake venom is composed of different proteins and enzymes where, all are serving a variety of purposes, such as intrusive with a prey's cardiac system or increasing tissue permeability so that venom is engrossed faster **(**Stefansson *et al,* 1990; Hati, *et al.,* 1992; Dennis, 1994; Kini, 1997; Gutierrez *et al*, 1997). The venom in some snakes like pit-vipers, affects virtually every organ system in the human body which can be a mixture of many toxins, including cytotoxins, hemotoxins, neurotoxins and myotoxins allowing for an enormous range of symptoms. The venom of a particular snake was considered earlier to be one kind only *i.e.* either hemotoxic or neurotoxic (Andriao *et al*, 2000; Bawaskar, 2004; Mohapatra *et al,* 2011).

Main composition of Snake Venom

The venoms produced in specialized oral glands of snakes are of highly toxic in their secretions because, these oral glands are connected to the salivary glands of other vertebrates. The venom can be considered as modified saliva that contains rich hydrolytic enzymes, which is a complex mixture of polypeptides, nucleases, peptidases, etc. that in turn helps to digest snake's prey. Besides, the chemistry of snake venoms is very much complicated in which, venoms are having 90% protein and most of these proteins in venoms are enzymes. There are about 25 diverse category of enzymes have been isolated from snake venoms, out of which nearly 12 categories of enzymes occur in all most all venoms; but on the contrary, all the toxic chemical compounds in snake venoms are not enzymes. Generally, snake venom will fall into two major categories *i.e.,* hemotoxic venom affects the circulatory system (heart and cardiovascular system) and neurotoxic venom affects the nervous system and brain. Additionally, snake venom is also used for other purpose like increasing the prey's uptake of toxins (Reid and Theakston, 1983; Shah, 2009 and Petras *et al*, 2011).

Since time immemorial, man continuously has been utilizing medicinal plants for basic preventive and curative health care through traditional medicinal system. In addition, phyto-chemicals and non-nutritive chemicals present in the herbs and fruits may have a protective efficiency against a host of diseases in the human biological system. The plant based products produces the active chemicals to have protection from various ailments in human system, nevertheless, there are some recent research reports which demonstrates that, many active group of phyto-chemicals can protect humans against some serious diseases. Hence, natural products, either as pure compounds or as standardized plant extracts can provide unlimited opportunities for new-fangled drug leads because of the unrivaled availability of diverse class of chemicals (Mors *et al*, 2000; Otero *et al*, 2000ABC; Perales *et al*, 2005; Sanchez *et al*, 2008 and Paul Reta *et al*, 2011).

Later on, the concept of 'ethno-medicine' has acquired significant attentiveness in its therapeutic approaches for various ailments and diseases. The therapeutic advancement via ethno-medicines is nothing but a medical anthropology that deals with the study of traditional medicines against ailments through its restorative mode of treatments. The traditional knowledge of ethnic groups and their practices have been verbally transmitted to subsequent generations since several years (Houghton and Osibogum, 1993; Kerns *et al*, 1999; Mebs, 2000; Alzate *et al.,* 2000; Alam and Gomes, 2003; Betti, 2004; Kokwara *et al*, 2005; Kodel and Jain, 2008; Jain and Singh, 2010 and John *et al*, 2011). The explosion of literature on ethno-medicines and their medicine formulations has been encouraged by an augmented awareness of the consequences of the forced displacement and acculturation of ethnic people. Besides, the recognition of health concepts as a means of sustaining ethnic identities and the search for innovative therapeutic drug and technologies are enforced to the public domain. The validation of conventional medicines and their claims of usefulness in

ethno-medicinal plant drugs along with the formulations are playing crucial role in management of poisonous snakebite. In addition, the evaluation of anti - venom properties displayed by the extracts of ethno-medicines and their active formulations are the real need of the day (Mors *et al*, 2000; Mebs, 2000; Fabricant and Fransworth, 2001; Soares *et al*, 2004; Samy *et al*, 2008; Meenatchisundaram *et al*, 2008; Makhija and Khamar, 2010).

Therefore, in the present study, the neutralization effect of the Snake venom was undertaken in ethno-medicinal plant drug formulations named as TMF (Tribal medicine Formulation) which is practiced by Tribal medicine men at Biligirirangana Hills, Karnataka. Since, the preliminary studies on physico-chemical, phyto-chemicals, anti-microbial and antioxidant activities are well reported on different candidate herbal medicines; the present study focused explicitly, on the efficacy of TMF in order to assess the Anti Snake Venom activity pertaining to Anti-PLA_2 and interaction of proteins between Snake venom and TMF drug in order to develop a most potent herbal drug.

MATERIALS AND METHODS

The present analytical study was carried-out at the department of engineering chemistry, Akshaya Institute of Technology, Lingapura, Tumkur-Koratgere Road, Tumkur - 572106, India. The survey was conducted at Biligirirangana Hills (Chamarajanagar district of Karnataka) to obtain first hand information's from Tribal Medicine Men (TMM). Subsequently, the interaction was held with TMM and the data on ethno-medicinal plants and Herbal medicine formulation for snake bite was collected (Fig. 1A-I).

Requirements

The materials and chemicals used for phyto-chemical analysis are Picric acid, α- naphthol, Benedict's reagent 5% Ferric chloride, % gelatin, 10% Sodium hydroxide, Alcohol, Biuret's reagent, Ninhydrine reagent, Lead acetate, NaOH, Conc.H_2SO_4.The chemicals used in the screening are of analytical grade. For Thin layer Chromatography, TLC sheet, TLC chamber, Micro pipettes, 1% Ninhydrine solution and solvents used are n-propanol and water.

Source of Venom

The Lyophilized Snake venom of *Naja naja* (common cobra) was purchased from Irula Snake Catchers Industrial Society Pvt. Ltd Chennai, Tamilnadu and was preserved at 2 to 8°C for future use.

Validation of Tribal Medicine Formulation

After obtaining the plant materials along with the raw details of medicine formulations from tribal healers, the samples were scientifically validated based on its physical characteristics in association with an authorized Ayurvedic practitioner (Fig. 1A-I). The standard protocols were identified and the methodology was employed in the present study was based on the descriptions of previous reports (Chaithra, 2013).

Preparation and processing of Ethno-medicinal Plant drugs and Tribal Formulation

The ethno-medicinal plants were procured based on the data base obtained from Tribal medicine men. The different parts of ethno-medicinal plants such as leaves, tubers, whole plant materials were separated accordingly and subjected for cleaning followed washing with tap water in order to remove all the dirt and unwanted particles prior to the drying process. Then, the processed materials of plants were subjected for shade drying for 3-4 weeks at room temperature (28 ± 1°C). The dried materials were then crushed into a coarse dry powder with a suitable mechanical blender and put through a sieve according the standard protocol and stored in hermetically sealed container.

Water extraction of Crude TMF drug

The crude extract from validated formula tion of TMF drug was prepared from through soxhlet extraction method. The powdered plant material was weighed (100g) accurately and uniformly packed into a thimble for extraction. The mixture was subjected for heating on a hot plate with continuous stirring at 40ºC for 15 to 20 minutes. Then, the water extract was filtered through Whatmann No.1 filter paper. The filtrate was subjected to dryness and the dried extract was kept in refrigerator at 4ºC for further analysis.

Thin Layer Chromatography

The sample (1mg-a speck) was dissolved in required amount of solvent system and the sample was introduced onto the TLC plate using a micro-capillary tube. The end of the micro capillary was dipped into the sample solution, then, flow of a small volume of the solution into the micro capillary was observed. The spots were made using the capillary onto the pencil lines on TLC plate and the specific care was also taken about the spots on the TLC plate was around 3 mm in diameter. The spotting solvent was allowed for a few seconds to evaporate and then, the TLC plate was introduced in the elution chamber keeping the side of sample spots at the bottom direction. The sample spots were maintained above the level of the elution solvent. The sample elution was allowed to a point where the solvent front is about 5mm from the top of the TLC plate. The spots were visualized on TLC plate under UV light or iodine or a series of chemical stains. The polarity of the solvent was adjusted if the retention factor (Rf) of the analyte is too large or too small. The Rf was calculated by dividing the distance traveled by the analyte by the distance traveled by the solvent. The ideal solvent gives the analyte with Rf of 0.3.

Gel Permeation Chromatography (Sephadex-G-75)

This experiment was performed in order to purify the extracts of TMF drug. The materials required for gel

permeation chromatography are column setup, spectrophotometer, sephadex-G-75, Saline and the chemicals used in the screening are of analytical grade. In permeation chromatography (also known as size exclusion chromatography) separation was done based on differences in the size and shape of the analyte molecules, which governs the analytes access to the pore volume inside the column packing particles. The smaller analytes can entered the pores more easily and therefore spend more time in these pores, increasing their retention time. On the other hand, larger analytes spend little if any time in the pores and were eluted quickly. All columns have a range of molecular weights that can be separated; if an analyte is either too large or too small it will be either not retained or completely retained, respectively. The analytes that are not retained are eluted with the free volume outside of the particles (Vo), while analytes that are completely retained are eluted with volume of solvent held in the pores (Vi). The total volume can be considered by the following equation, where Vg is the volume of the polymer gel and Vt is the total volume. This can be appraised using the following formula.pounds as described by Raaman, 2006.

The sephadex G-75 was suspended in saline solution, incubated for overnight. A column was packed in a vertical glass column apparatus (1.8cm dia, 30cm height). The column was prepared by pouring the slurry of swollen gel particles in saline into the tube to obtain a bed height of 26 to 28 cm. Two or three column volumes of eluent (the same saline) were passed through the column at 2ml/5min to stabilize and equilibrate the gel bed. 1ml of the sample was loaded from the top of the column without disturbing the gel bed. The column was eluted at 2ml/5min and 17 fractions were obtained with different test tubes.

Antimicrobial Activity

Microorganisms

The microorganisms were procured from authorized hospital, Tumkur, (India) and the pathogenic microorganisms were selected for study includes bacteria, *viz., Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Salmonella* sp. and *Bacillus subtilis.* The bacterial strains were cultured and maintained on "Muller- Hinton (MH) agar medium at pH 7.4±0.2) and incubated at 37±2° as per the standard procedure.

Disc diffusion assay

The antimicrobial screening was performed using Disc diffusion method and MH agar was used for bacteria. The base plates were seeded with the standard size of bacterial, (1×108 CFU/ml) and sterile filter paper discs (6mm in diameter) were impregnated with 100µl of each of the extract (10mg/ml concentration) to give a final concentration of 1mg/disc, left to dry in vacuum to remove residual solvent, which might interfere with the determination. The extract discs were then placed on the seeded agar plates and each extract was tested in triplicate along with standard streptomycin (1mg/disc) for bacteria. The plates were kept at 4°C for 1h for diffusion of extract and incubated at 37±2°C for 24 h, respectively. The zone of inhibition (IZ) or depressed growth of microorganisms was measured for TMF drug and calculated using the standard formula (Andrews, 2001).

Antioxidant activity

The radical scavenging activities in the extracts of TMF against 2, 2-Diphenyl -1-picryl hydrazyl radical (Sigma - Aldrich) were determined by UV-Spectrophotometry at 517 nm. Radical scavenging activity was measured by a slightly modified method as described elsewhere.

Hydroxyl Scavenging Assay

The required reagents were pipette-out to the test tubes containing the PBS. The total volume of the reaction mixtures was made to 1ml. The reaction mixtures thoroughly mixed using vortex mixer and subjected for incubation at 37^0C in water bath for 1 hour. To this, 1ml of 1% TBA was added to all the test tubes after incubation. Then, the reaction mixtures were boiled in the water bath for 20 minutes and allowed to cool and subsequently, acetone was added to stabilize the colour and read the absorbance at 535 nm.

TBARS Assay

In the experiment, 50µl of ferrous sulphate and 100 µl of ascorbic acid were added to each test tube which contains 50µl linolenic acid. The volume was made to 0.5ml with TBS. The antioxidants were mixed in the different ratios. The Lipid peroxidation was induced by Fe^{2+} ascorbate system in lipid membrane micelles. The reaction mixture contained erythrocyte ghost membrane micelles in TBS (tris pH 7.4) $FeSO_4.2H_2O$, ascorbate and various concentration of the antioxidant i n a final volume of 0.5 ml. the reaction mixture was incubated at 37°C for 60 min. The treatment of oxidized lipid with 1ml of 1% TBA was incubated in hot water bath for 15 min, which may results in the formation of coloured complex and allowed to cool. Then, 2ml of acetone was added to stabilize the colour. The peroxidation was estimated as thiobarbutric acid reactive substances (TBARS) at 535 nm. The lower value of absorbance indicates, higher the inhibition of lipid peroxidation. Then, BHA and vitamin E at 400µM was used as known standard antioxidant where it quenched and inhibited the lipid peroxidation.

Anti-venom activity

Partial purification of Snake venom protein by Gel filtration chromatography

The venom of snake, *Naja naja* sample was obtained from the authorized sources as per the procedure. The venom sample was purified using column chromatography loaded with the silica gel. A known amount

(30mg) of *Naja naja* venom was dissolved in buffer and loaded to column of bed volume; 94.2 and fraction was eluted at the flow rate of 2ml per 5 minutes. The bed volume; 11/2 of protein samples was eluted for each tube the optical density was measured at 280 nm.

Purification of sPLA$_2$ from *Naja naja* venom

The sPLA$_2$ isolated from *N. naja* snake venom was subjected for purification to homogeneity as described in the previous report (Samy *et al.,* 2012). The venom, (110 mg) was fractionated on CM-Sephadex C-25 column (1.4 x 120 cm) using phosphate buffer of different molarities (0.02 - 0.4) and pH (7.0 - 8.5). This was then eluted into thirteen major fractions and the fraction of NN-I-PLA$_2$ was chosen for further purification. The lyophilized fraction NN-I-PLA$_2$ (25 mg) was rechromatographed on CM-Sephadex C-25 column (1.6 X 30 cm) which was pre equilibrated with 0.1 M phosphate buffer, pH 7.0. The protein was eluted using phosphate buffers of 0.1 M, pH 7.0 and 0.15 M, pH 7.5, respectively. This was eluted into NN-I-PLA$_2$ (a) and NN-I-PLA$_2$ (b) fractions. These 2 fractions were dis- solved separately in 0.1 M NaCl and loaded on to se- phadex G-50 column (1.4 X 92 cm) which was pre equil- ibrated with 0.1 M NaCl and eluted with the same sol- vent. Both the peaks were homogenous and showed PLA$_2$ activity. The homogeneity was checked by SDS- PAGE (Laemmli, 1970).

Sodium Dodesyl Sulfate-Poly-Acrylamide Gel Electrophoresis (SDS-PAGE)

In the study, SDS-PAGE was carried out according to the standard method of Laemmli, (1970). The homogeneity of purified sPLA$_2$ samples were checked using 12.5 % resolving gel. The protein bands were visualized by staining the gel with 0.25 % Coomassie brilliant blue R-250.

Estimation of Protein

Protein content of the TMF sample was determined by employing the method of Lowry *et al.* (1951) using BSA as protein standard. The aliquots of 0-1 ml standard BSA was taken in clean and dry test tubes, 5 ml Lowry's reagent (98 ml of 4 % sodium corbonate + 1 ml of 2 % copper sulphate + 1 ml of 2 % sodium potassium tartarate) was added. After 15 min, 0.5 ml of Folin and Ciocalteu's (FC) phenol reagent (1:1 diluted with water) was added and left for 30 min. The OD was measured at 660 nm.

Structure of Phospholipase A$_2$ (PLA$_2$)

The Phospholipases A$_2$ (PLA$_2$) is commonly found in mammalian tissues as well as insect and snake venom. The venom from both snakes and insects is largely composed of melittin, which is a stimulant of PLA$_2$ and due to the increased presence and activity of PLA$_2$ resulting from a snake or insect bite, arachidonic acid is released from the phospholipid membrane disproportionately. As a result, inflammation and pain occur at the site of biting. The Phospholipases A$_2$ (PLA$_2$s-EC 3.1.1.4) are inflammatory enzymes that releases fatty acids from the second carbon group of glycerol. This particular phospholipase specifically recognizes the sn-2 acyl bond of phospholipids and catalytically hydrolyzes the bond releasing arachidonic acid and lysophospholipids. The cyclo-oxygenases, arachidonic acid are modified into active compounds upon downstream modification called eicosanoids. Eicosanoids include prostaglandins and leukotrienes by which are categorized as inflammatory mediators.

Phospholipase A$_2$ Activity assay

This experiment was carried out by employing the method described in the previous report (Bhat and Gowda, 1989 and Bhat *et al.,* 1991) which involves the measurements of free fatty acids liberated by the enzyme catalyzed hydrolysis of egg-phosphotidyl choline. A set of 7 cleaned and dried vials were taken; to this, 50µl of phosphotidyl choline and 50-200ug of concentrated venom samples were added. The PLA$_2$ enzyme was calcium dependent. So 20µl of calcium was added followed by the addition of Tris HCl buffer (pH 7.5) and made up to 1ml. Then, 200ul of petroleum ether was added and incubated for 1 hour at 37^{0}C. After incubation, the reaction was arrested by adding 500ul of doles mixture and 1000ul of petroleum ether and then, centrifuged at 500xg for 5 min. The supernatant (0.5 ml) was collected. Further, to this 500ul of chloroform: petroleum ether was added followed by the addition of 500ul of cobalt nitrate reagent. The mixture was subjected for centrifuged at 1200xg for 10 min and the supernatant of about 0.5ml was collected. Finally, to this 1-nitroso 2-naphthol solution of about 750ul and 2000ul of methanol was added. Then, the OD for the sample was read at 540nm spectro-photometrically using glass cuvettes.

Anti-hemorrhagic Study

This analysis was conducted in order to find out the Snake venom activity through Myonecrosis and lung hemorrhage activity, the Mouse lung tissue and also the muscle tissue layer was collected and added with Saline buffer to one layer and the cell death did not occur. Then the other layer was added with Snake venom and the cells started dying by forming tumors. Then the other layer was added with the Snake venom sample along with the TMF extract sample and there was decreased activity of the enzymes which were present in the venom and this showed the activity of TMF extract over venom in neutralizing the effect of the venom.

Anti-hemorrhagic Test

The old fertile eggs were obtained from a local hatchery were subjected for incubation till day 4 at 38°C and the eggs were cracked on day 4 into Clingfilm hammocks following a standard method and incubated further till day 6. The discs of 2 mm diameter cut from

filter paper (Whatman No. 2) were impregnated with a Standard Hemorrhagic Dose (SHD) of venom *Naja naja* (3g/1.5) alone or venom and various concentrations of TMF extract. The discs were then placed on the yolk sac membrane over a major bilateral vein and left for 3h for hemorrhagic corona to form and the corona was measured with a ruler. The control experiments were performed with buffered saline solution used to prepare the extract and venom solutions. The readings were taken in triplicate and the abolishing of haemorrhage at a minimum concentration was recorded as the Minimum Effective Neutralizing Dose (MEND).

RESULTS

The database on Tribal medicine formulation (TMF) was obtained from the traditional healers during survey and followed by interactions (Fig.1A-I). The habitual view of *Naja naja* and milking of venom was documented from the authorized sources (Fig. 2A-D). The results generated in the present study have been represented in the tables, figures and graphs respectively. The validated Tribal medicine Formulation (TMF) was documented with the help of authorized Ayurvedic Medical practitioner (Table 1). Further, the physiochemical characteristic features of the TMF drug sample are revealed hereunder.

Physico-chemical analysis

The plant sample was collected and dried and then the different tests were carried out in order to find the physical and chemical properties. Thus the Total ash, Acid insoluble ash and Water soluble ash was obtained (Table 2).

Phyto-chemical analysis

The outcome of qualitative phyto-chemical analysis of aqueous and solvent extracts such as ethanol, methanol, acetone, petroleum ether, ethyl acetate and hexane extracts of TMF drug were analyzed. The proteins were present in cold and hot water extracts followed by ethanol solvent extracts whereas, the protein was not shown in methanol, ethyl acetate, petroleum ether and hexane extracts respectively. The carbohydrates are present in hot water, cold water and absent in all the other extract. The phenolic content was present in all the extracts. The presence of phyto-chemicals were present in the various solvent extracts of the TMF drug were noticed. The presence of active phyto-chemicals in TMF drug extracts may be responsible for all the biological activities such as antimicrobial, antioxidant activities etc (Table 3).

Antimicrobial activity

The zone of inhibition at both gram positive (*Staphylococcus aureus*) and gram negative (*Escherichia coli, Pseudomonas aeruginosa* and *Salmonella* sp.) bacterial species was observed in different solvent extracts of TMF drug. The antibacterial potency of TMF drug was assessed by the presence or absence of inhibition zone in diameter. The growth of bacteria like *S. aureus, E.coli* and *S. typhi* were considerably inhibited by the cold water extract followed by other organic solvent extracts. The activity of the cold water extract was higher than that of the other extracts, similarly the ethanol extract exhibited better activity. *E.coli* was greatly inhibited by Ethyl acetate. Besides, the cold water extract of TMF drug showed considerable antimicrobial activity against *S.aureus* and *Salmonella sp.* with more zone of inhibition and on the contrary, the less zone of inhibition was observed in *P.aeruginosa* and *E.coli* (Table 4). The result revealed that, the TMF drug contained scores of diverse group of bioactive agents which are connected with antimicrobial properties in this formula. This active principle could be an authentification about the practice of drug and would be most useful in identification and control to adulterations of the raw drug.

Antioxidant activity

The radical scavenging assay via ABTS method gave the measure of antioxidant activity of the ethyl acetate extract and petroleum ether determined by the decolorization of the ABTS. +, through measuring the reduction of the radical cation as the percentage inhibition of absorbance at 734nm. The effects of ethyl acetate and petroleum ether extracts of TMF drug on ABTS free radical scavenging activities was assayed at concentrations of 400 µg/ml. The significant ABTS free radical scavenging activity of 77 ± 0.4 and 78±0.5 was evident in this TMF extract and the standard reference taken was Gallic acid (Table 5). This result shows that significant removal of free radicals from the cells which are causing damage to the cell connected to the apoptosis.

Thin layer chromatography

This was done in order to purify the TMF drug solvent extracts and the bioactive compound moved on the stationary phase was measured as well as retenti on factor was also calculated. There were two to three bands of whole TMF drug extract at variable Rf values were observed. The Rf value of the ethanol extract, petroleum ether, and ethyl acetate showed 0.923, 0.958 and 0.970 respectively and other extracts showed less Rf value as compared to these extracts (Fig-3A&B). The bioactive compound moved on the stationary phase was measured and the retention factor was calculated.

Protein extraction through Gel permeation chromatography

The fractions were collected at the rate of 2ml/5min for 1hour 30min from the column G-75. The fractions were collected in different test tubes and read at 280nm spectrophotometrically and plotted the graph of O.D vs. elution time. Further, the different fractions of extracted snake protein showed a prominent peak and were significantly superior over other small peaks

respectively. Further, the spectra of active principle was shown present in the TMF protein (Fig-4&6).

Protein profile of TMF drug on SDS-PAGE

The Protein profile of TMF drug on SDS-PAGE, TMF formula (10μg) was performed in association with low molecular weight markers (12μg) loaded on gel, electrophoresis carried at 80V for 5% stacking gel, 40V for 12.5% resolving gel. After electrophoresis the gel stained with Coomassie brilliant blue R_{250} stain for 8h. Lane 1=TMF drug (15kDa) and lane M=Low Molecular weight markers, Ovalbumin (45kDa), Carbonic anhydrase (29kDa), Aprotinin (6.5kDa) respectively (Fig. 5).

Protein profile of Snake venom on SDS-PAGE

In the profile of Snake venom proteins, the fractions were collected at the rate of 2ml/5min for 90 min from the column G-75. The fractions were collected in different test tubes and read at 280nm spectrophotometrically and plotted the graph of O.D vs. elution time (Fig. 7).

The separation of venomous proteins on SDS-PAGE was carried-out in association with low molecular weight markers (10μg) loaded on gel, electrophoresis carried at 80V for 5% stacking gel, 40V for 15% resolving gel. After electrophoresis the gel stained with Coomassie brilliant blue R_{250} stain for 8h. lane M=Low Molecular weight markers, Lane 1=Venomous Protein. The final impression was found to be low molecular weight proteins (Fig. 8).

Interaction between Proteins of TMF drug and Snake venom

The plant protein and venom protein showed significant interaction and they were almost concurrent each other. Thus, the TMF drug protein neutralized the effect of the venom protein (PLA2). The fractionation of crude aqueous (cold) extract of TMF yielded two peaks with peak 2 being the major one with maximum inhibition of TMFV-PLA2 (complex) enzyme activity (Fig. 9).

Anti Snake venom Activity

The plant protein and venom protein showed significant interaction and they were almost concurrent each other. Thus, the TMF drug protein neutralized the effect of the venom protein (PLA_2). The fractionation of crude aqueous (cold) extract of TMF yielded two peaks with peak 2 being the major one with maximum inhibition of TMFV-PLA_2 (complex) enzyme activity. The spectra of snake venom protein, dose dependent inhibition of sPLA_2s and Spectrum of venom protein with the plant extract (Fig. 9 & 10). The study reveals that, the partially purified bioactive principle of TMF drug neutralized the PLA_2 and interaction of Proteins of both snake venom and TMF. Inhibition of enzymes of snake venom protein of *Naja naja* was considerably significant (Table 6).

Snake venom neutralization effect in active principle of TMF drug

The spectra of snake venom protein, dose dependent inhibition of sPLA2s and Spectrum of venom protein with the plant extracts were assessed along with venom neutralization effect in association with TMF drug formula. The study reveals that, the partially purified bioactive principle of TMF drug neutralized the PLA_2 and interaction of proteins of both snake venom and TMF. The inhibition of enzymes of snake venom protein of *Naja naja* was considerably significant (Fig. 10 & 11).

In the study, the efficacy of TMF drug and their active constituents showed in neutralizing the pathophysiological action of phospholipase A2 (PLA_2) purified from *Naja naja* snake (dose dependent). These active compounds were found to neutralize the *Naja naja* venom PLA2 induced toxic effects with a variable extent. However, the TMF drug purified protein was found to be superior in neutralizing the toxic effect of tested PLA_2 (Table 7 and Fig. 11).

Anti-hemorrhagic Test

In this analysis, the Snake venom activity through Myonecrosis and lung hemorrhage activity were noticed. The Neutralization of the haemorrhagic activity was estimated by mixing a fixed amount of venom with different amounts plant extracts. The plant extract–venom mixture was incubated at 37°C for 1 h and 0.1 ml of the mixture. The mouse lung tissue and muscle tissue layer added with Saline buffer showed no cell death or even desertion in the tissue system was observed (Fig 11 & 12). The second layer added with snake venom showed the cells started dying by forming tumors whereas in case of the third layer added with the Snake venom sample along with the active principle of TMF drug was found to be very effective in decreasing the activity of the enzymes which were present in the snake venom and this confirms the efficacy of TMF drug extract over venom in neutralizing the effect of the snake venom (Table 7).

DISCUSSION

The present research describes, scientifically uninvestigated/ignored and less known ethno-medicinal plant drugs showing chemical constituents of natural origin with possible mechanisms of anti-venom activity. The ethno-medicinal plants were procured from traditional healers of study area, B.R.Hills in raw conditions. Then the TMF drug formulation was validated scientifically for its authentification. Further, the extract was verified for its physico-chemical, phyto-chemical, antibacterial, antioxidant and anti-venom activity respectively. The phyto-chemicals of diversified categories were analyzed qualitatively in the extracts of TMF drug apart from its pharmacognostic status . The aqueous and solvent extracts were tested for both antimicrobial and antioxidant properties followed by studies on neutrali-

zation effect of TMF drug in the selected snake venom (*Naja naja*). The parameters of physico-chemical such as, total ash, acid insoluble ash and water-soluble were justified the pharmacological parameters. The preliminary phyto-chemical study was done for the detection of secondary metabolites such as, alkaloid, flavonoid, glycoside, phenol, saponin, terpenoids and tannin. The presence of active metabolites was defensible with respect to practice of these ethno-medicinal plant drugs by the traditional healers. The phyto-chemicals evolved in the TMF drug formula can be considered as viable parameters, which will go a long way for prescribing a dependable standard to the raw drugs. Moreover, phyto-chemical and elemental analysis provides importance information which may be help in authentication and adulteration for quality control of row material (Alam *et al.,* 1990; Chang *et al*, 2002; Khandelwal *et al*, 2006; Patel *et al*, 2010).

In addition, the TMF drug formula also substantiate the presence of significant physico-chemical properties and indicated that, occurrence of active compounds with novel mechanisms of action that in turn encourage a rational approach for antivenom activity. The presence of vigorous phyto-chemicals and their active principles obsessed that, these active constituents exists in the different fractions of extracts to adjust some specific chemical characteristics which have strong inhibitory effect against lethality and myotoxic activities induced by the snake venom. The patients with rigorously envenomed were given the crude decoction and a black distinctive stone was fixed on the snake bite surface by the healers who strongly believed that the black distinctive stone could neutralize the poisons and act as an antidote. Though this mode of treatment practiced widely in the study area, proper evidence on the information from these healers was missing from the scientific point of view. However, the present findings TMF drug treatment also corroborate with the previous reports of Mc Donald *et al*, 2001; Chang *et al*, 2002; Khandelwal *et al*, 2006; Abika bothya *et al*, 2011).

The recent scientific strategies for the evaluation of natural product with specific biological activities require the implementation of well-defined screening process. Therefore, the results of present investigation have attributed that, the TMF drug extracts possesses potent antimicrobial activity against bacterial types. Correspondingly, antioxidant assay through ABTS, TBARS and the hydroxyl scavenging method and were shown to be potential in the extracts of TMF drug and found to be significant. The TMF drug is being used in indigenous system of medicine as well as local residents either as single drugs or in combination for the snake bite and related ailments. Hence, the active fractions of TMF drug showed significant scavenging of the ABTS radicals at very low concentrations of the drug. This is in agreement with the previous studies (Pietta *et al*, 1998; Lee *et al*, 2000; Kessler *et al*, 2003; Huang *et al*, 2005; Margaret *et al*, 2012).

The TMF drug extracts exhibited better performance against bacteria like *Staphylococcus aureus, Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa*. The higher antibacterial activity of the ethanol and ethyl acetate extracts may be due to the greater solubility of the extract in the above said organic solvents. But, the aqueous extracts with mixture of several natural constituents were found to be more effective against *S. auerus*. The antibacterial activity has been attributed to the presence of some active phyto-chemicals present in the extract of TMF drug. This also indicates the bioactive molecule is associated with water can able to perform superior activity, whereas, in organic solvents, only the identified or specified compounds will be dissolved and showed better activity against pathogenic microorganism with collective constituents of natural condition. The specified extracts with cold water, ethyl acetate and petroleum ether were most active and showed high activity against common drug resistant microorganism used in the study. This may be due to the greater stability of the bioactive principle in the solvent over a longer period of time (Sunitha *et al.,* 1996; Lai and Roy, 2004; De lima *et al*, 2006).

The antioxidant activity of natural products from plant was reported that, the particular concentration of plant extract is expressed in dry weight of extract for each fixed dosage of the assay mixture will be of core focus. Therefore, the IC_{50} value represents the concentration of test extract or compound where the inhibition of the test activity reached its optimum level. This may be due to the addition of potassium per sulphate thus leads to formulation of free radicals. Hence, the extract of TMF drug exhibited dose dependant inhibition on superoxide anion.

The significant and effective neutralization was noticed between both protein of *Naja naja* and TMF drug. Initially, the interaction between two proteins was found to be most effective, however the interaction was found to little differ with two prominent peaks from proteins of both plant and snake venom and interestingly at the end again these two proteins were found two interact effectively (Hassham, *et al*, 2000; Borges *et al*, 2000 & 2001; Ticli *et al*, 2005; Cavalcante *et al*, 2007; Chethan kumar *et al*, 2010). The fractionation of crude aqueous (cold) extract of TMF drug yielded two peaks with peak 2 being the major one with maximum inhibition of TMF-$VPLA_2$ enzyme activity. Then, production of tissue destructive free oxygen radicals occurs due to inflammation of snake venom PLA_2 which aid in disruption and supportive towards formation of highly reactive lipid peroxides. There were some studies available relating to isolation, purification and characterization of active bio-molecules in the medicinal plants, which are decisively acting as effective inhibitors in reducing the local tissue damage and toxicity of snake venom (Girish *et al.,* 2005, Chatterjee *et al.,*

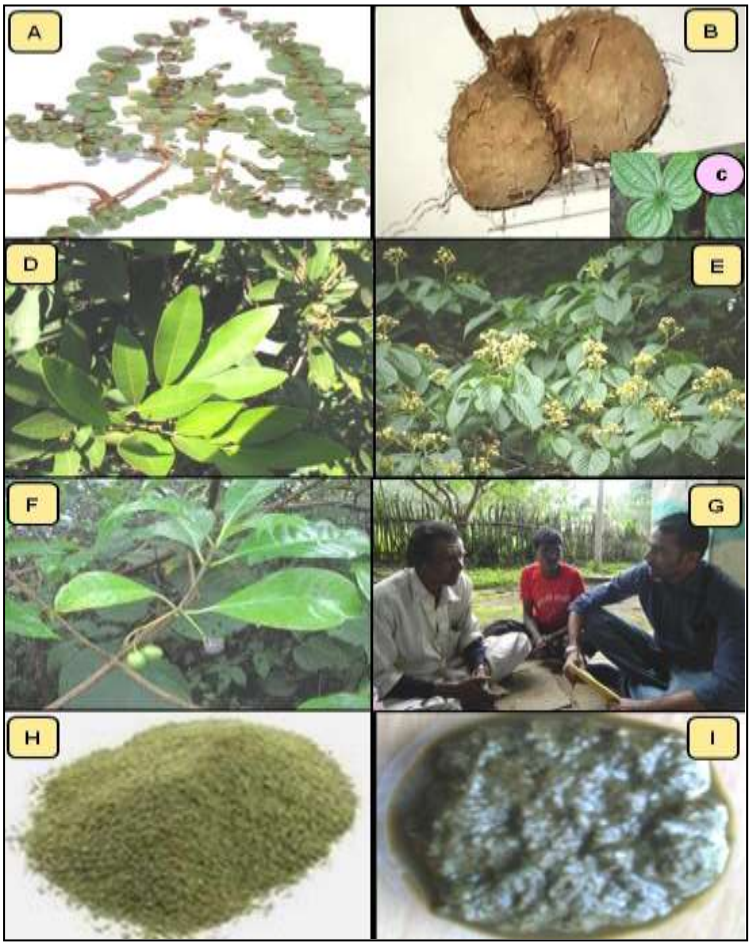

Figure 1A-I: Ethno-Medicinal Plant Components in Tribal Medicinal Formulation (TMF)

A: Leaves of *Andrographis serphyllifolia* **, B:** Tubers of *Dioscorea hispida,* **C:** Leaves of *D. hispida* (Inner view), **D:** Leaves of *Glycosmis mauritiana,* **E:** Leaves of *Nothopodytes nimoniana* **, F:** Leaves and stem of *Rauwolfia densiflora* , **G:** Interaction with Tribal Healers, **H:** Powder of Tribal Medicine Formulation, **I:** Paste/slurry of Tribal Medicine Formulation.

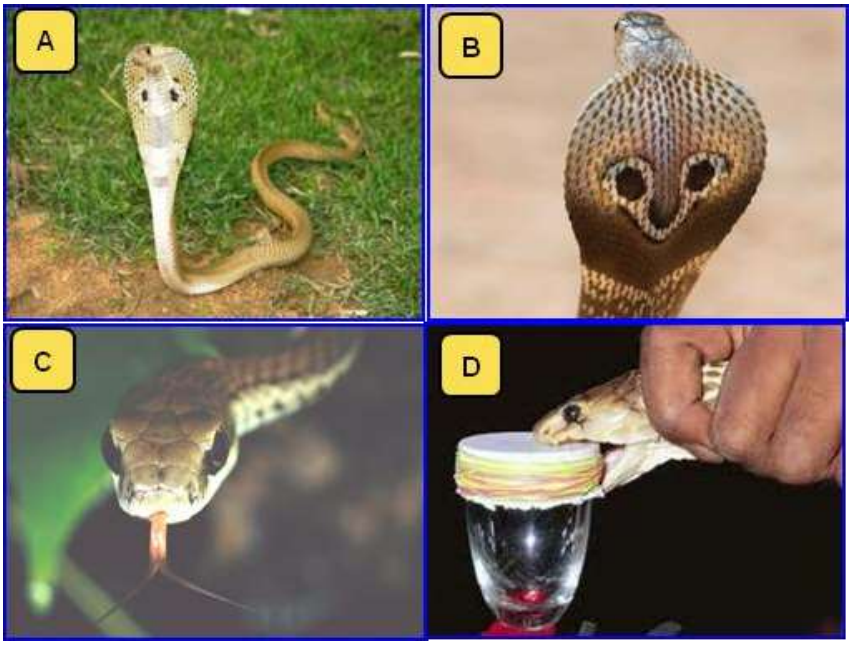

Figure 2: A-D. A: Naja naja - habitual view, B: Showing spectacle mark on the hood of N. naja, C: Showing Bi-fid tongue, D: Milking of venom from Naja naja

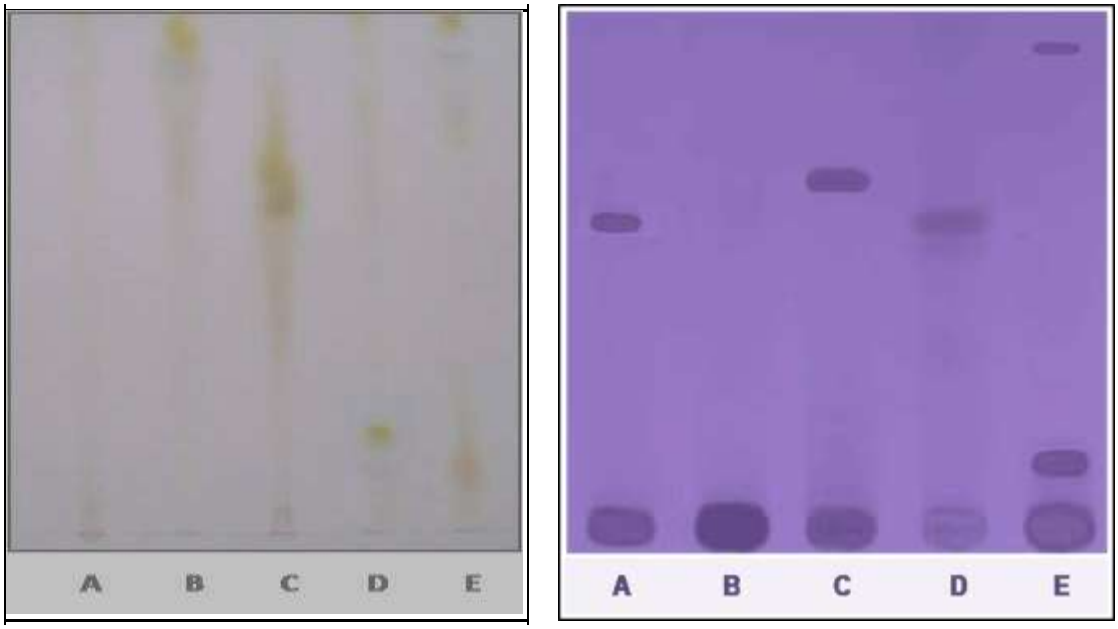

Figure 3: A: Thin layer chromatography showing (fluorescent spots) presence of bioactive constituents in the extracts of TMF drug formula 3B: The TLC Chromatograms of the individual components of TMF drug extracts (Observed under ultra violet light at 366 nm)

Table 1: Validated Tribal Medicine formulation (TMF) and its components practiced for Snake-bite and anti-inflammatory related ailments at Biligirirangana Hill Tracts, Karnataka.

Sl. No.	Ethno-medicinal plants with Vernacular Name.	Family	Plant parts used	Quantity (powder) (g/kg)	Validated Quantity of TMF (g)*
1	Andrographis serphyllifolia Vahl. **(A)** Vr. Name: *Kasinasara*	Acanthaceae	Whole plant	20	**(A)** 20+ **(D)**15+ **(G)** 25+ **(N)** 25+ **(R)** 15+ **(TMF) ADGNR = 100g**
2	Dioscorea hispida Dennst. **(D)** Vr. Name: *Noolana hambu*	Dioscoreaceae	Tubers	15	
3	Glycosmis mauritiana (Lam) Tanaka. **(G)** Vr. Name: *Orrange berry*	Rutaceae	leaves	25	
4	Nothapodytes nimoniana, Blume. **(N)** Vr. Name: *Durvasane mara*	Icacinaceae	Leaves	25	
5	Rauwolfia densiflora Benth & Hook. **R)** Vr. Name: *Snake root*	Apocynaceae	Leaves	15	
DOSAGE, DURATION AND MODE OF TREATMENTS OF TRIBAL MEDICINE FORMULATION					
Paste of TMF	It is applied on affected part in snake bite and Scorpion bite with few drops of Honey and Lime juice for wounds and infected area.			**Duration** Apply paste at wound area & cover with a thin cloth 3times/week	
Decoction of TMF	Ground & juice boiled with warm water & swallowed internally for snake bite problems. Decoction with warm water/ goat milk for inflammation and related ailments			**Duration** One tsp two times a day for 8 days.	

2004). Additionally, the polyphenols from the aqueous extracts of the TMF drug formula may perhaps took part during complete inhibition of the lethality of *Naja naja* venom. The efficacy of TMF drug protein has been responsible to neutralize snake venom may be due to specificity and active sites possessing anti-lethal effects (Mores *et al.,* 2000; Alam & Gomes, 2003; Arce *et al*, 2009; Kadiyala, 2011). The interaction between both protein of venom and TMF drug was considerably noteworthy which conceivably influenced by the presence of active secondary metabolites in the drug formulation. Then,

Table 2: Physico-chemical analysis extracts Tribal Medicine Formulation (TMF)

Sl. No	Parameters	Results
1.	Total Ash	23.45%
2.	Acid Insoluble	3.78%
3.	Water soluble	5.63%

Table 3: Phyto-chemical analysis in the different extracts Tribal Medicine Formulation (TMF)

Tests	Cold water (g%)	Hot water (g%)	Ethanol (g%)	50 % Ethanol	Methanol	Ethyl acetate	Petroleum ether	Hexane
Protein	0.36	0.23	0.16	0.28	Nil	Nil	Nil	Nil
Ascorbic acid	0.18	0.36	0.01	0.02	Nil	Nil	Nil	Nil
Total Sugars	0.04	0.05	Nil	Nil	Nil	Nil	Nil	Nil
Polyphenols	0.61	0.83	0.01	0.42	0.39	0.88	0.99	0.19
Flavonoids	0.72	0.92	0.01	0.32	0.22	0.77	0.23	0.18

Table 4: Antimicrobial activity in the different extracts Tribal Medicine Formulation (TMF)

TMF drug Samples at 300 uG/DISC	Cold water	Hot water	Ethanol	50% Ethanol	Methanol	Ethyl acetate	Petroleum ether	Streptomycin 20 ug/disc
Escherichia Coli	**15±1.2**	15±0.7	8±0.2	7±1.2	11±0.5	14±0.6	14±1.2	22±1.2
Pseudomonas aeruginosa	**14±0.7**	11±0.3	9±0.8	10±0.2	12±0.8	12±0.4	13±0.9	26±0.7
Staphylococcus aureus	**17±0.7**	13±1.2	6±0.4	9±0.45	14±0.8	12±0.5	12±0.7	19±0.5
Salmonella typhi	**18±0.8**	9±0.6	6±0.7	9±0.4	14±0.9	11±0.6	13±0.5	19±0.5

Table 5: Antioxidant activity in the different extracts Tribal Medicine Formulation (TMF)

TMF drug Samples at 300 ug/ml conc.	Cold water	Hot water	Ethanol	50% Ethanol	Methanol	Ethyl acetate	Petroleum ether	Hexane	STD ascorbic acid 400 uM
TBARS	51±1.2	61±0.7	63±0.9	70±1.2	65±0.5	76±1.1	74±1.2	43±0.5	90±1.0
OH scav-enging	68±1.2	71±1.0	66±0.6	66±1.2	60±1.5	72±0.1	73±0.8	30±1.5	84±0.7
ABTS	56±1.8	69±1.7	66±0.4	54±0.4	64±0.9	77±0.4	78±0.5	45±0.8	79±0.5

Table 6: AntiPLA2 activity of Naja naja snake venom in the different extracts Tribal Medicine Formulation (TMF)

TMF drug Samples at 200 uG/ml	Cold water	Hot water	Ethanol	50% Ethanol	Methanol	Ethyl acetate	Petroleum ether	Lipoic acid 50uM
Anti PLA_2	**66 ±1.2**	66±1.9	45±0.8	54±0.9	61±1.2	43±0.2	45±1.2	92±0.7

the active constituents like terpenoids, flavonoids, polyphenols might have also contributed significantly as anti-venom potential in the TMF drug. Therefore, multi-functionality of TMF drug has built a depiction in the capacity of such chemical substances that stick to proteins which further facilitate obstruction the functions of many macromolecules (Vishwanath and Gowda, 1987; Alam *et al*, 1994; Gowda *et al*, 1997; Camey *et al*,

Table 7: Inhibition of sPLA2 Activity in different samples

Sample	Specific activity (n moles/mg/min at 37 ºC)	IC_{50} (µg/ml)
Naja naja	138.0	79.2±2.3
Human synovial fluid	89.5	57.30±1.65
Human pleural fluid	92.75	66.23± 1.18

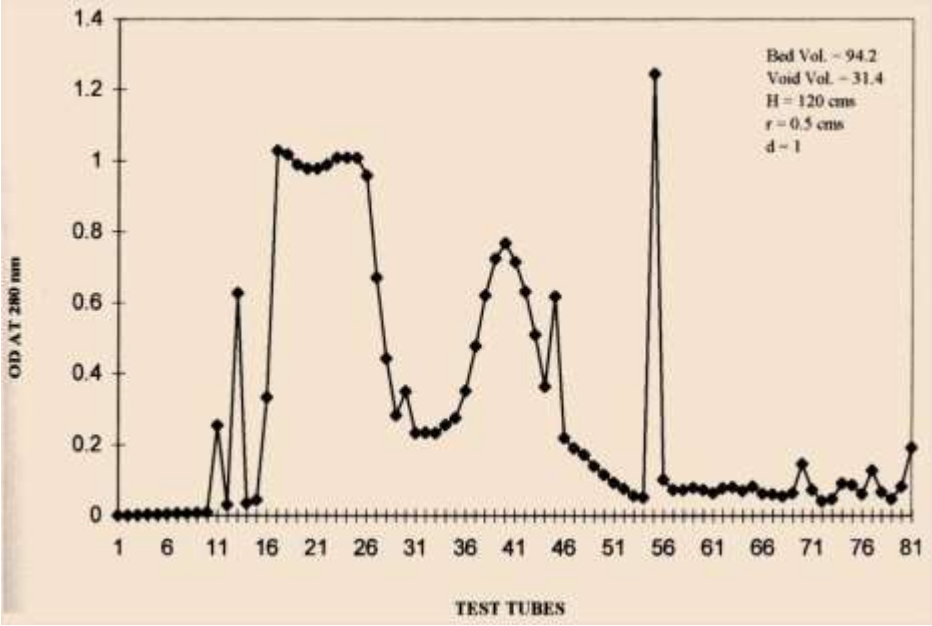

Figure 4: Showing Protein spectra of TMF drug extract

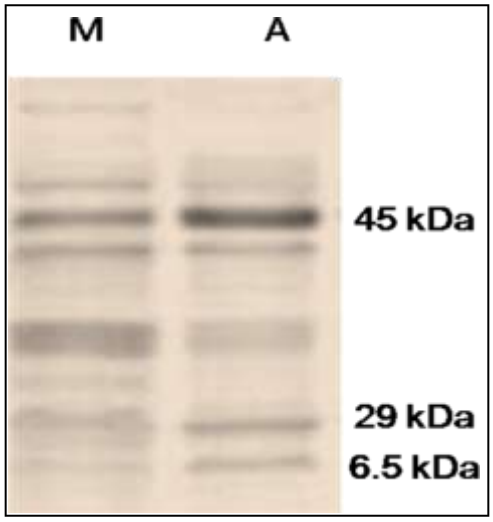

Figure 5: Separation of proteins from TMF drug by SDS-PAGE M) Protein ladder; A) TMF drug proteins.

2002; Nunez *et al*, 2004; Shirwaiker *et al*, 2004; Sakda *et al*, 2005; Chaterjee *et al,* 2006; Petras *et al*, 2011). However, it can be suggested that, these vigorous constituents are responsible for observed protection.

Later, the TMF drug used for the traditional treatment of snakebite were fractionated by silica gel chromatography. The active fraction of TMF drug neutralized the venom activities through plausible interaction of both proteins. The binding due to overlapping of both proteins indicates that, there might be distinct active sites which could establishes the interaction between these two proteins and the mechanism of action admits the neutralizing effect of the enzymes present in the venom (Ramar *et al*, 2008). Thus, the anti-PLA_2 and anti-protease activity was effectively carried out in neutralizing the effect of the enzymes like PLA_2 and protease present in the venom. Hence, these ethno-medicinal plant drugs can be used as a life saving drug by conducting the supplementary clinical trials (Santhosh *et al*, 2004; Pithayanukal *et al*, 2004 & 2005; Ermila *et al*, 2005; Arce *et al*, 2009; Jhon *et al*, 2011).

However, the research findings can be ascertained based on the veracity of the herbal assertions in the TMF drug holds a good promise for the development of

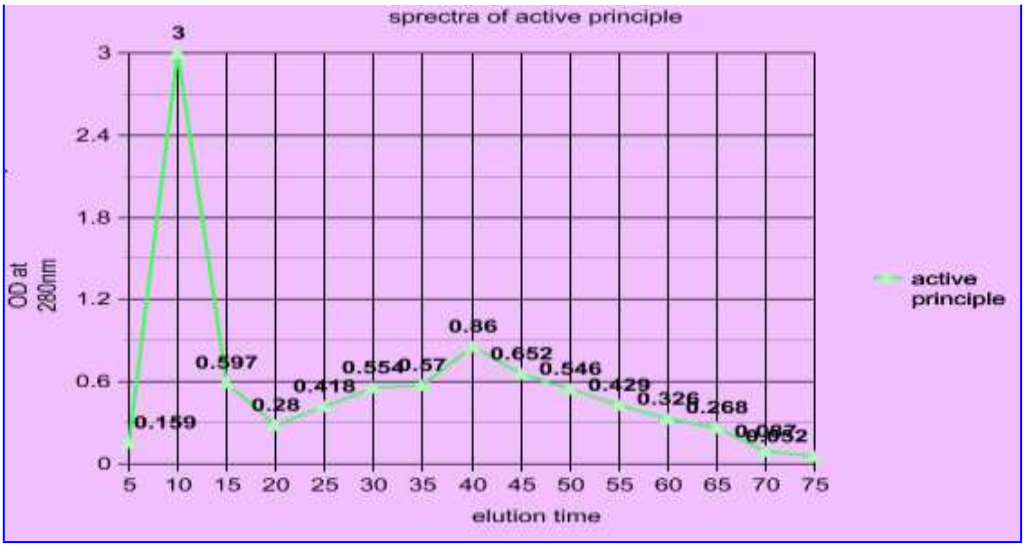

Figure 6: Spectra of Active principle from the extract of TMF drug

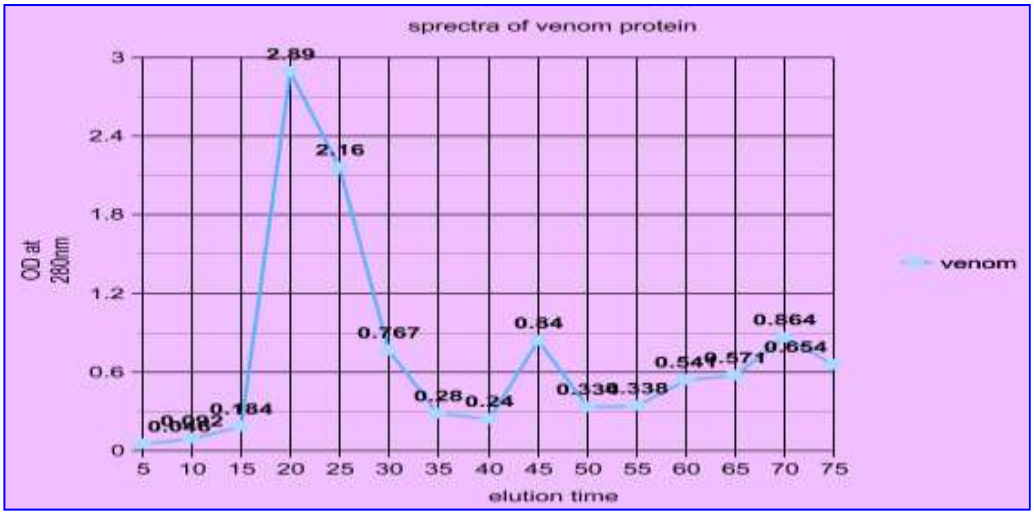

Figure 7: Spectra of snake venom protein of Naja naja

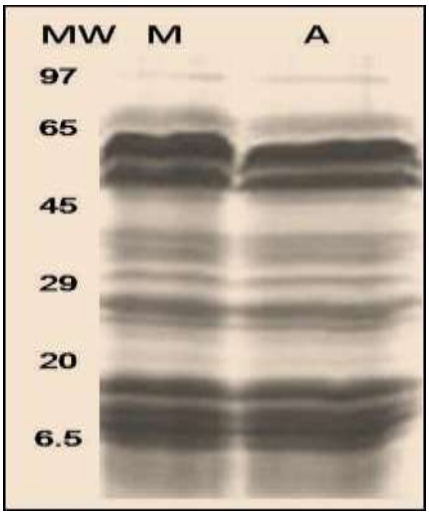

Figure 8: Separation of the venomous proteins by SDS-PAGE M) Protein ladder; A) *Naja naja* venom proteins

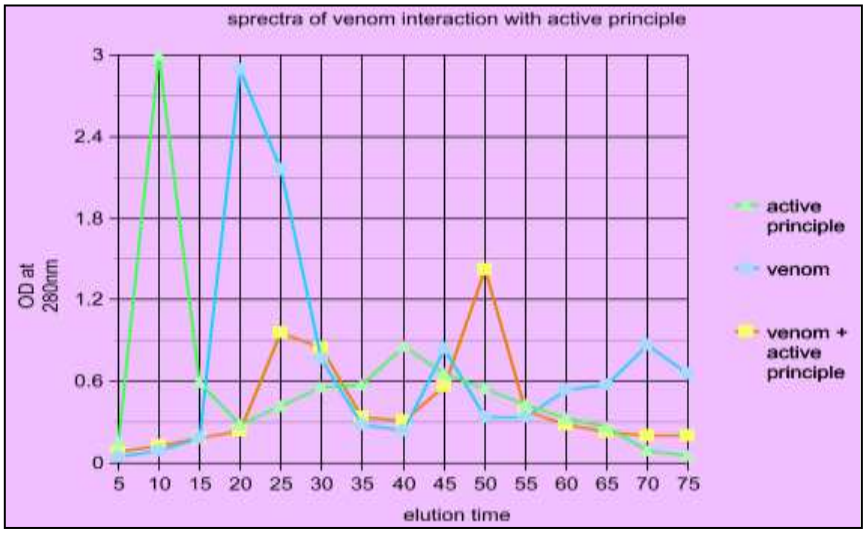

Figure 9: Interaction between proteins of both TMF drug and snake venom

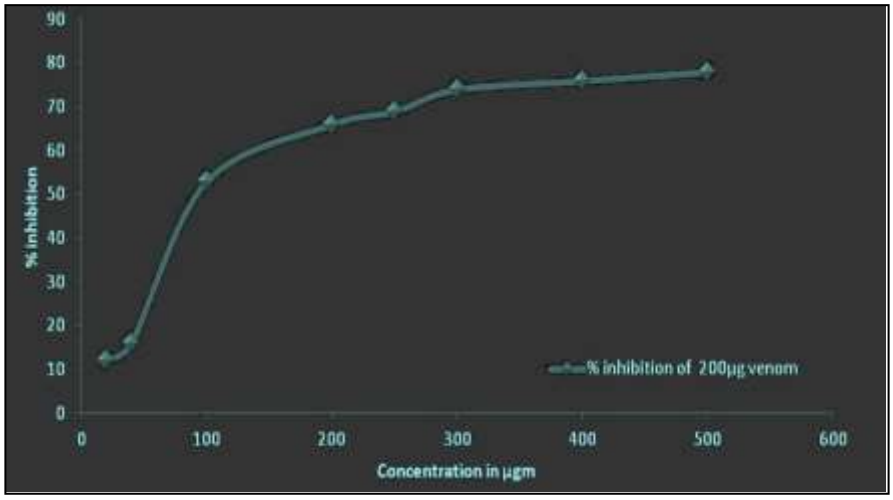

Figure 10: Spectra of Snake venom protein, Dose dependent inhibition of sPLA2s (Dose-dependent inhibition of PLA_2 with Cold water extract of TMF)

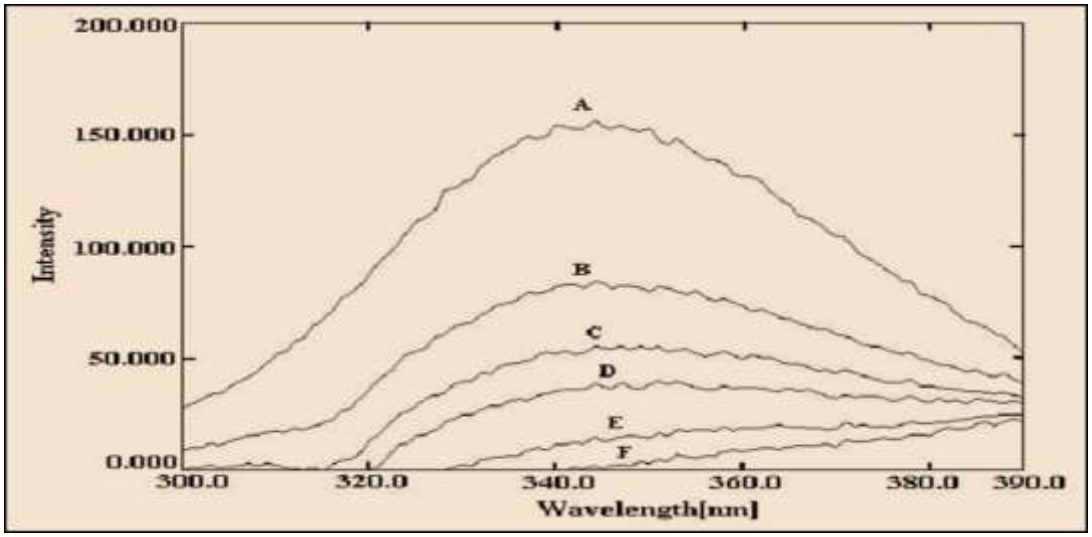

Figure 11: Spectrum of Snake Venom protein and different extracts of TMF extract A- PLA2 Enzyme alone, B-PLA2 with 40 µg/ml CWTMF, C-PLA2 with 80 µg/ml CWTMF, D-PLA2 with 120 µg/ml CWTMF, E-PLA2 with 200 µg/ml CWTMF, F-PLA2 with 500 µg/ml CWTMF

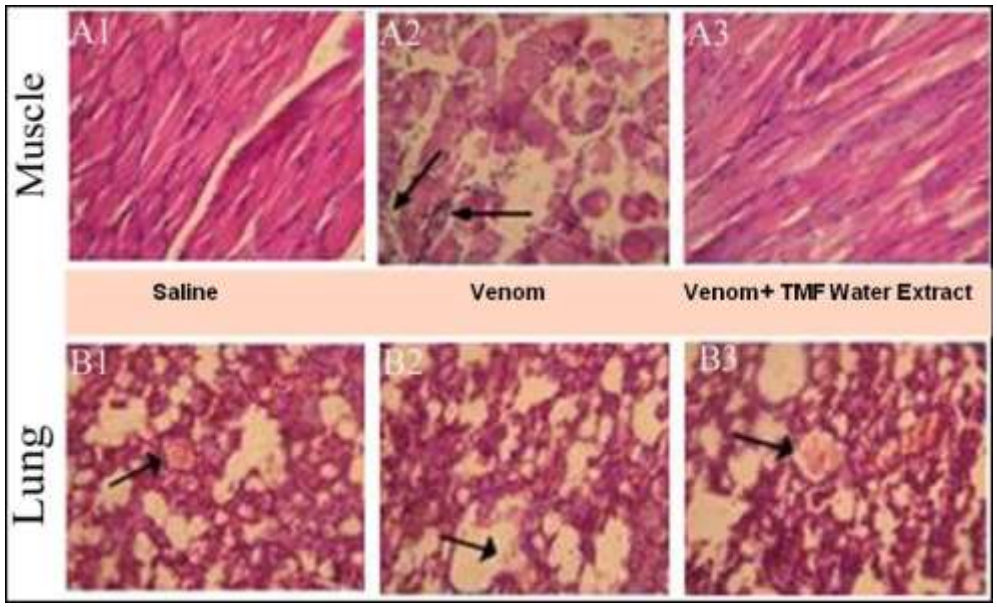

Figure 12: Myonecrosis and Lung hemorrhage: Light Micrograph of Mouse Tissue sections (histology)
A[1]: No Cell death and desertion of tissue system, **A[2]**: Cell death and desertion of Tissues with tumours,
A[3]: No cell death and tumours due to the activity of TMF drug.
B[1]: No necrosis and perceptible cells, **B[2]**: Cell death followed by necrosis,
B[3]: organized cells with no necrosis sustained due to TMF drug mixture with venom

novel anti-snake venom drug in the pharmaceutical outlook. The combination of herbal compounds with anti-venom may also be a good prospective as well as effective in neutralizing Snake venom (Andriao-escarso *et al*, 2000; Daduang *et al*, 2003; Daduang *et al*, 2005; Girish *et al,* 2006; Meenatchisundaram *et al,* 2008 & 2009; Petras *et al*, 2011). The accomplishment of proper herbal formulations along with efficacy in relation to counteractive appraises against snake bites are not yet known properly, hence, the comprehensive exploration has been elicited in the current study.

The snake bite victims in rural parts of India, mostly depends on the herbal antidotes practiced by traditional healers which is an alternative treatment available at that particular situation. This is because of inadequate and non-availability of primary health centers. Therefore, the present study has been focused on the traditional approaches in TMF drug for its anti-snake venom efficacy, which might be a stepping stone in the establishing the future therapy against snake bite treatment and management. It was observed that, the ethanolic and cold water extract of TMF drug when given to the mice after they received snake venom of *Naja naja* significantly increased mean survival time and protection fold but could not protect mice from death when used alone and the results were found to be better when the cold water extract was used at higher dose (Yao *et al*, 2004; Ushanandhini *et al,* 2006; Hussain *et al*, 2011). This could be possible due to inactivation or precipitation of active venom components by the cold water extract of TMF drug. The TMF drug extracts effectively neutralized the snake venom inducing lethal, haemorrhagic, coagulant and anti -coagulant activity both in vivo and in vitro. Besides, the active fraction effectively antagonized the *Naja naja* venom inducing patho-physiological changes which could further induced haemorrhagic, coagulant and anticoagulant activities (*both in vitro and in vivo*) were significantly neutralized by TMF drug fractions. The extracts of TMF drug possess energetic compounds with different chemical configuration were reported to be capable of interacting with peptides and proteins (enzyme) of snake venom (Gene *et al.,* 1989; Alkofahi *et al.,* 1997; Da-Silva *et al.*, 2005; Nishijima *et al.*, 2009; Carvalho *et al.*, 2013; Alam, 2014). In addition, the presence of active fractions in TMF drug confirms that, these vital compounds could act as powerful inhibitors of the hemorrhagic and clotting activity, probably due to interaction with proteins of purified fraction of TMF drug and which could facilitates, thrombin-like enzymes, respectively. This TMF extract could be a promising source of natural inhibitors, such as flavonoids, tannins and ploy phenols which probably act by forming complexes with metal ions and proteins that inhibit the action of serine proteases involved in blood coagulation disturbances and metalloproteases responsible for hemorrhagic processes and enzymatic activity of phospholipases A_2 (Oliveira *et al*, 2005; Kumarappan *et al*, 2011; Fernandes *et al*, 2011) .The mechanism of action of the TMF extracts/plant compounds are still not very apparent and they may be attributed to the blocking of receptors-structure prone to chemical attack and may block the active site of the snake venom.

CONCLUSION

The significant neutralization effect of Tribal Medicine formulation (TMF) against the venom of *Naja naja* was achieved, which is probably due to presence of various

vigorous phyto-constituents apart from dynamic profile of protein present in the TMF formula. The mechanism of action during neutralization through interaction played an imperative role in inhibition of PLA_2 activity in the snake venom. The above observations confirmed that, the extracts of TMF drug (comprising of the mixture of different components of candidate ethnomedicinal plants) possess intoxicating snake venom neutralizing capacity and could potentially be used as an adjuvant for antivenin therapy in case of snakebite envenomation, especially against the local effects of cobra venoms. The present result forms the basis for thorough examination of the active fractions with clinical trials for further investigation in the potential discovery of new natural bioactive lead molecule as dri v-ing force for snake bite victims.

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