CHAPTER 7

Summary, Conclusion and Recommendations
7. SUMMARY, CONCLUSION AND RECOMMENDATIONS

The healing of wounds often deviates from normal course, under-healing, over-healing or failure of wounds. The research on drugs that increase wound healing is a developing area in modern biomedical sciences. Several drugs obtained from plant sources are known to increase the healing of different types of wounds. The ethnomedicinal plants are coming into prominence because of the overuse of conventional medicines such as antibiotics which has resulted in development of resistance with many infectious organisms. Thus, the preparation of traditional medicine formulation can be more effective than conventional medicines and their non-toxic nature reveals that they can be administered over long periods.

In the current study, ethno-pharmacological informations were gathered through semi-structured questionnaire, individual and group interactions with traditional medicine men by following the standard procedure. The raw concoction of ethno-medicinal plant drugs was collected from local traditional healers through concrete interactions. The procured plant materials were authenticated with standard floras and taxonomic experts subsequently, the details on Traditional medicine formulation (TMF) was collected explicitly for methodical substantiation. The ethnomedicinal plant drugs were selected were of (EMP) viz, *A. serphyllifolia* (Acanthaceae), *D. hispida* (Dioscoreaceae); *G. mauritiana* (Rutaceae); *N. nimmoniana* (Icacinaceae) and *R. densiflora* (Apocynaceae) and Tribal Medicine Formulation (TMF) drug were procured in its raw status. Further, the TMF was validated scientifically with authorized ayurvedic practitioner and was subjected for chemo-typing and characterization studies. The ethno-medicinal plants were processed according to WHO bioassay guidelines and subjected for pharmacological, phyto-chemical evaluations, respectively.

In the beginning, the plant materials were subjected for physico-chemical parameters such as ash values, foreign organic matter, moisture contents and extractive values respectively. In addition, fluorescence analysis for the ethno-medicinal plants samples was carried out. The proximate analysis reveals that, the value of acid insoluble ash and water soluble ash were found to be significantly lesser correspondingly, the solvent extraction rate was significantly higher as compared to the values of water
soluble ratio. The amount of total ash present in the samples of *A. serphyllifolia* was 55.25%, the acid insoluble ash present in the leaves was 60.15%, the value of water soluble ash extractive was found to be 78.40%, the foreign organic matter present in leaves was 24.50% and moisture content present in the sample was 45.01%. Similarly, the amount of total ash present in the samples of *D. hispida* was 56.75%, the acid insoluble ash present in the leaves was 44.16%, the water soluble ash extractive value was 63%, the foreign organic matter present in leaves was 58.40% and moisture content present in the sample was 52.52%. In the samples of *G. mauritiana*, the contents of total ash (44.56%), acid insoluble ash (82.27%), water soluble ash (98.77%), foreign organic matter (40.20%), moisture content (42.20%). For *N. nimmoniana*; the contents of total ash (52.28%), acid insoluble ash (65.18), water soluble ash (70.66%), foreign organic matter (39.10%), moisture content (35.10%) and *R. densiflora showed* the contents of total ash (40.22), acid insoluble ash (55.72%), water soluble ash (72.50%), foreign organic matter (60.30%), moisture content (22.22%) were recorded.

Further, the screening of selected ethno-medicinal plant drugs and Tribal medicinal formulation clearly indicate the presence of maximum classes of active phyto-constituents is present in the extracts of both EMP and TMF drugs respectively. The phyto-chemical screening of aqueous extract and solvent extracts of EMP and TMF demonstrated the presence of alkaloids, flavonoids, saponins, tannins, gums & mucilages, coumarins, terpenoids, steroids, glycosides, phyto sterols, fixed oils and fats, anthraquinone and phycobalamin. The occurrence of active metabolites was also suggested that, the synergistic actions of these active metabolites exerted the significant pharmacological potentialities. The screening evaluation reveals that; the ethno-medicinal plants; *A. serphyllifolia* and *D. hispida* did not contain cardiac glycosides and coumarins while, *G. mauritiana*, *N. nimmoniana* and *R. densiflora showed* occurrence of active metabolites like alkaloids, tannins, glycosides, saponins, terpenoids etc. Additionally, the exclusive metabolites like phycobalamin and anthraquinone were also confirmed qualitatively in diversified extracts of both EMP and TMF drugs respectively. The phyto-chemical tests were best answered by the aqueous extracts of TMF than that of solvent extracts of EMP. In addition, the amount of total phenolic compounds in all tested plant extracts was higher than the TMF drug. The ranking order of five plant species from point of view of antioxidant (phenolic
compounds) amounts was as follows: *R. densiflora* > *N. nimmoniana* > *G. mauritiana* > *A. serphyllifolia* > *D. hispida*.

Subsequently, in the cytotoxic assay, the brine shrimp mortality was found to be increased with increase in test concentrations. The noxious and momentous action (p=0.001) was displayed to the nauplii of brine shrimp at active fractions of both EMP and TMF drugs. Later, the antimicrobial evaluation was carried-out by employing both Gram-positive, *Bacillus subtilis* and *Staphylococcus aureus*; Gram-negative; *Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pneumonia*. As a whole, the active fractions of EMP and TMF showed perceptive affects against Gram positive bacteria, whereas, in case of Gram-negative organisms, they were considerably susceptible solvent extracts such as, Ethanol, Methanol, petroleum ether compared to aqueous extracts of both EMP and TMF. The zone of inhibition was found to be most effective at methanol and ethanolic extracts of EMP and TMF drug which was compared with standard antibiotics for instance, Flucanazole (1.0 mg/disc) and Ampicillin (1.0 mg/disc). Similarly, the excellent activity was recorded with petroleum ether extract followed by aqueous fractions. In total, the fractions of both EMP and TMF drug were found to be effectual against all pathogenic microorganisms. The high degree of (inhibition zone) antibacterial activity was noticed against *E.coli* by ethanolic extracts of *A. serphyllifolia* (14±0.2), *D. hispida* (14±0.6), *G. mauritiana* (16±3.5) *N. nimmoniana* (14±1.5) and *R. densiflora* (16±1.0). Similarly in TMF, the zone of inhibition was (19±0.2) significantly superior than other solvent extracts. For *S. aureus*, the ethanolic extract exhibited radical increase in zone of inhibition at *A. serphyllifolia* (13±0.3), *D. hispida* (16±2.5), *G. mauritiana* (19±1.5) *N. nimmoniana* (15±1.0) and *R. densiflora* (21±3.0) and the maximum inhibition (23±2.0) was recorded.

Correspondingly, in the antifungal activity, *A. flavus* showed efficient antifungal activity for ethanol extracts EMP drugs viz., *A. serphyllifolia* (19.6±0.6), *D. hispida* (16.9±0.5), *G. mauritiana* (17±0.4) *N. nimmoniana* (16±0.3) and *R. densiflora* (15.5±0.4). On the contrary, the TMF Methanol drug extract showed proficient activity in *A. niger* (21±0.3), *A. flavus* (22.1±0.6), *F. oxysporum* (24.8±0.11) and *R. stolonifer* (26.1±0.5) respectively. Further, the maximum dilution factor or else lowest concentration of the active fractions of the drug employed to suppress the microbial growth called Minimum Inhibitory Concentration (MIC), which is an important tool
to identify the drug resistant pathogenic microorganisms against different standard antibiotics. The results reveals, ethanolic extracts of TMF showed outstanding activities both in MIC (21.9 μg/ml) and MBC (58.7 μg/ml) in S. aureus followed other extracts against all the pathogenic organisms and were also significantly superior over all the EMP drugs. Similarly, the aqueous and ethanol extracts of EMP drugs (Glycosmis mauritiana) showed least MIC value 26.5μg/ml and 29.3 μg/ml against E. coli respectively. On the contrary, the highest MIC (56.2 μg/ml) and MFC (116.6 μg/ml) activity was observed in aqueous extract of A. serphyllifolia by A. flavus, like-wise, in petroleum ether extract of N. nimmoniana, MIC (57.4 μg/ml) and MFC (114.3 μg/ml) activity was recorded in A. niger. Whereas, petroleum ether extract of R. densiflora showed considerably significant MIC (59.9 μg/ml) and MFC (119.8 μg/ml) at R. stolonifer. In all, the aqueous fractions of both EMP and TMF crude fractions were exhibited maximum activity when compared with different solvent extracts on all the bacterial and fungal cultures tested.

The fractions of different extracts of EMP and TMF drug was subjected for scavenging free radicals through DPPH and ABTS approaches. The free radicals were down regulated and scavenged effectively at the active fractions of EMP drugs which were compared with Ascorbic acid used as standard. The significant DPPH scavenging activity in methanolic extracts of EMP drugs, A. serphyllifolia (18.42 ± 0.05 EC50 μg/ml), D. hispida (18.90 ± 1.42 EC50 μg/ml), G.mauritiana (29.12 ± 0.56 EC50 μg/ml), N. nimmoniana (16.66±0.85 EC50 μg/ml) and R. densiflora (31.55 ± 0.65 EC50 μg/ml) respectively were recorded. Where as in case of TMF the % DPPH activity was found to be 90% at 150μl concentration and the activity was increased with increase in concentrations of TMF. Similarly, in ABTS assay, the increase in concentrations (60-150 μg/ml) facilitated the superior activity from 15- 64%. Similarly, the enhanced DPPH radical scavenging was noticed with respect to increased solvent fractions of the EMP drugs. In total, the better activity of radical scavenging (90.85%) in TMF drug was recorded at150μg/ml. In all, it was confirmed that, the radical scavenging ability of both EMP and TMF drug was found to be most prospective in regulation of the free radicals by donating electrons. Therefore, the most promising activity was obtained for FRAP assays on the contrary, the low down expressions were noticed for DPPH assay respectively. The qualitative estimation of total phenol content showed, the highest total phenolic levels were detected in the
extract of *R. densiflora* and the lowest in the extract of *D. hispida*. The amount of total phenolic compounds in all tested plant extracts was higher than the TMF drug.

The total reduction capability showed that, the extracts of both EMP drugs, the IC\(_{50}\) values of reducing activities of aqueous fractions revealed the order of activity as: 0.36% in *A. serpyllifolia*, 0.45% in *D. hispida*, 1.17% in *G. mauritiana*, 1.28% in *N. nimmoniana* and 1.12% in *R. densiflora* were recorded. Later on, TLC chromatograms was developed using appropriate solvent system, the bioactive compound moved on the stationary phase was measured and the retention factor was calculated. There were two to five bands of whole TMF drug extract at Rf values of 0.32 and 0.44 in *A. serpyllifolia*; 0.52 and 0.26 in *D. hispida*; 0.59 and 0.23 in *G. mauritiana*; 0.34, 0.14, 0.16, 0.62 and 0.95 in *N. nimmoniana* similarly, in case of *R. densiflora* 0.76 and 0.85 were present. The extracts showed variable Rf values as compared among these extracts.

As the wound infection is a major cause of morbidity and mortality in most of the patients, the plant based traditional medicine extracts may be useful in preventing infection that leads to high risk of sepsis. The generated results demonstrate that the aqueous extract formula of various parts of the ethno-medicinal plant drugs would be capable of promoting wound healing activity. Further, the study on fractionation of active components and the mutual effect of these TMF extract is performing as machinery on infecting microbial species which may provide a better understanding of the various issues of wound related ailments towards effective management in the process of wound healing.

The effect of active fractions of Tribal Medicine Formulation (TMF) was employed to analyze the wound healing potentialities via excision and incision models. The assessment on wound healing tests was performed for drug formulation using the simple ointment base (B.P) as control along with reference hypothesis, Nitrofurazone ointment 0.2% w/w by way of excision wound model. The momentous effect from the topical therapy at the dosage of 10 and 15% w/w ointment of EMP and TMF drugs exhibited superior (P<0.05) action on wound contraction as compared to the control treatment administered to the groups of animals of excision wound model. Correspondingly, the striking effect of the active fractions of the drug on tensile strength in the incised wound after 10\(^{th}\) day of wound healing process was also found to be extremely significant.
In the incision wound model, increase in tensile strength was found to be noteworthy (515 ± 2.42) after 10 days of wound due to treatment with test formulation. Besides, the active principle of TMF drug was triggered the healing process to attain contraction which substantiate the traditional practice of the crude drug by herbal practitioners at BRT. The dose dependent effect was noticed by the TMF drug on wound recovery followed by ethanolic fractions emphasizing on tensile strength after the termination of the wound healing phases. Subsequently, the ethanolic fraction of TMF drug over dead space wound model exhibited enhanced effect on granuloma weight followed by breaking strength which was correlated to level of hydroxyproline. In the histological evaluation, the animal group treated with TMF drug elicited the formation of collagen which facilitates wound contraction. Later, the defence related enzymes like, superoxide dismutase and catalase were showed significant activities which might have contributed to the existing antioxidant potentials in the system. Thus, the re-epithelialisation duration was considerably reduced during the phases of wound contraction and the treatment with TMF drug was found to be significantly superior over all other treatments including control. The TMF drug of both doses showed better performance in activating the wound repair process which further facilitated closure of the wound area with solid layer by overwhelming the scar tissue in the dermis region followed by reorganization of epidermis layer.

The wound contraction was exercised by the active principles of TMF drug of both crude and ethanol which were positive in both wound models. In both cases, the different phases of wound healing was accelerated favourably which reduces the actual period of wound contraction through their explicit actions on mechanism of action induced by the TMF drug. The wound healing process in excision model was set in motion at the topical application of TMF drug at 10 and 15% w/w and overall expression was found to most significant (P<0.05) on all the days healing process. The active fractions of ethanolic extract of TMF drug showed remarkable effect on wound closure through regaining the tensile strength of the wound which was superior over control treatment. The measurements of the tensile strength have been picturized based on period of epithelialization. It was evident that, the crude fractions of TMF drug were considerable promising at both the wound models which substantiate the practice of the drug by local traditional healers.
The formulated Tribal Medicine extracts was tested for anti-proliferative activity against HeLa cancer cell lines. The inhibition concentration as observed in the 24h duration of trypan blue assay. The cells show positive ‘Hoechst assay’ as the DNA has acquired the Hoechst stain and the same is seen under the fluorescence microscope. The results analysis for MTT assay indicated that the cell viability decreases with increase in concentration of the drug. The inhibition concentration value (IC50) for the MTT assay at 24h shows to be 5.1µg/ml and for 48h at 5.4µg/ml. The positivity of MTT assay can be observed through the formation of purple colour crystals. The outcome of mamarian cancer analysis showed that, the methanolic fractions of TMF drug exhibited profound activities over tested cells i.e., MCF-7 which was demonstrated through MTT assay. The index of cytotoxicity and cell viability were critically examined at dose dependent actions of the drug with respect to different exposure periods. Finally, the efficacy of TMF drug extract in terms of the cytotoxicity indexes showed a gradual increase of cell death effect had been achieved setting out on increased concentrations, reaching up to 80 to 90% of cytotoxicity for the MCF-7 cell lines.

The Apoptotic assays showed that TMF drug both ethanolic and crude fractions induced mitochondrial depolarization in MCF cells leads to 20-30% cell death followed 40-50% Cell Death by Trypan Blue Assay at lowest exposure periods of lowest concentrations. Further, the susceptibility of Mammarian cancer cells has been influenced by Tribal medicine formulation. The significance of these observations and recommendations in the light of previous studies with asynchronous population of MCF-7 cells has also been discussed.

The protein profile of the TMF drug on SDS-PAGE, was demonstrated using a specific amount of TMF formula (10µg) in association with the markers consisting of low molecular weight (12µg) laden on the gel electrophoresis. The experiment was performed at 70-80 V for 5% stacking gel and 12.5% resolving gel at 50V. After the processes, the protein profile was observed by means of a particular stain (Coomassie brilliant blue) which comprises Lane 1=TMF drug (15kDa) and lane M=Low Molecular weight markers, Ovalbumin (45kDa), Carbonic anhydrase (29kDa), Aprotinin (6.5kDa) respectively. Similarly, the separation of venomous proteins on SDS-PAGE was carried-out in association with the markers of (10µg) low molecular weight, which was laden on the gel and performed the electrophoretic experiment by
setting the parameters 70-80V stacking gel followed by resolving gel (15%) at 50V. The assessment showed that, the final impression was found to be low molecular weight proteins.

The significant and effective interaction was noticed between both protein isolated from biological resources. Initially the interaction between two proteins was found to be most effective, however the interaction was found to 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. Correspondingly, the partially purified bioactive principle of TMF drug neutralized the PLA₂ and interaction of Proteins of both snake venom and TMF. Inhibition of enzymes of snake venom protein of *Naja naja* was considerably significant. The presence of active lead molecules was 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 PLA₂. The significant neutralization effect of Tribal Medicine formulation (TMF) against the venom of *Naja naja* is probably owing to occurrence of diverse range of vigorous phyto-chemicals in the TMF formula which played an imperative role in inhibition of PLA₂ activity.

In the Anti-hemorrhagic analysis, the concoction of TMF drug and snake venom complex (0.1ml) was subjected for incubation at 37°C for 1h prior to analysis. Further, the different active fractions of TMF drug was employed to analyze the fate of hemorrhagic activity which demonstrates neutralization effect in the course of integration of snake venom sample with active principle of TMF drug at variable concentrations was observed. The mouse tissue sections of Myonecrosis and Lung hemorrhage reveals, that, 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. 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. Therefore, the active principle of TMF drug may possibly use as an adjuvant for anti-venom therapeutics, especially against the local effects of cobra
venoms. The present result forms the basis for selection of plant species for further investigation in the potential discovery of new natural bioactive compounds.

**Conclusion with possible Recommendations**

The generated results indicate that, further objectives can be carried out with this TMF drug to identify the lead/chief bioactive constituents responsible for the profound biological activities. Besides, based on the pharmacognostic status of the TMF drug, a novel drug can be formulated based on this TMF validated drug explicitly for snake bite apart from wound healing and related ailments. This will definitely facilitate the mankind in attaining a better, healthy, safe and sound life style. Further convoluted work is necessary to know-how the apparent mechanism of action relating to inhibition of snake venom. Further, detailed clinical studies in this direction are required to potentiate this claim in human beings.

The use of herbal remedy of natural origin for burn wounds is common in tropical countries such as the Philippines. Tribal Medicine Formula (TMF) is traditionally applied to burn wounds for effective, instantaneous and continued relief by the tribal community at B.R. Hills and surrounded area. This research investigated the healing potential of the TMF drug on burn wounds using mice model in the treatment. For possible exploration, different grounding aspects and protocols/procedures were employed with crude, methanolic/ethanoic and reference ointment treatments respectively. These were compared to the commercially available nitrofurazon (NF). The generated results suggests that, TMF drug and associated ethno-medicinal plant drugs, regardless of preparation procedure, were found to be most effective in treating wounds and their types which was compared with NF as reference treatmet. The percent wound contraction, re-epithelialization time, and histological features of the wound treated with TMF dug extracts was real-time approach when they were comparable to the reference and control.

However, this investigation may serve as a stepping stone for future research on the biological and pharmacological activities in the extracts of EMP and TMF drugs. In addition, the outcome of the study clearly confirms that, the practice of TMF drug in Tribal medicine system for wound healing has been substantiated. Hence, the pharmacologist can harness the potentials in the ethno-medicinal plants and in the TMF extracts of ointments for the treatment of contemporary diseases relating to
wound and associated ailments such as skin cut, skin infection, tissue damage etc. The ethno-medicinal plants and formulation certainly which are the real gift from the nature having traditional knowledge, provides excellent novel raw material towards better management of wound and other multiple ailments. Moreover, the result of the present study emphasizing on ethno-medicinal plant drugs (extracts) screens the soluble active fractions in the development of safe, cost effective and an acceptable wound healing herbal formulation, which is validated appropriately and the efficiency has been proved scientifically. Further, this TMF drug formulation can perform as an alternative and efficient surrogate medicine that possibly will even barter the other type of modern wound healing medicines.

On the other side, it is quite obvious that the high market demand of such plant derived medicines will build a pressure on our natural resources that might lead to the habitat loss, habitat degradation and over harvesting and exploitation of these medicinal plants resulting in a major threat to our biodiversity. Hence, a well thought road map should be designed for the proper consumption as well as maintenance of our valuable natural resources. This in turn, will not only provide affordable and effective plant based curative molecule(s) for treatment of various ailments but will preserve our indigenous knowledge as well. Still, the outcome of the present study may show the way to future scope of research with respect to purified fractions of TMF drug and associated ethno-medicinal plant drugs for biological and pharmacological activities using biophysical and clinical approach to know how the actual drug metabolism in the biological system. In addition, the reported experimental results can provide a concrete and legitimate platform for designing newfangled herbal drug comprising of multi-herbal concoction exhibit most prospective biological activities which can combat against multiple ailments.