CHAPTER 6

DISCUSSIONS

6 Discussion

Plants are an integral component of folk medicine, and their role in pharmacotherapy is invaluable as they harbour an "n" number of compounds with pharmacological significance. Identification and authentication of a plant is the first step in herbal product research, as adulteration occurring as a result of variation in geographical conditions, faulty collection, an unskilled workforce, identification by vernacular names, and collection time can be avoided, thereby ensuring a reproducible quality of herbal medicine [74]. The first stage in determining the identification and level of purity of herbal materials is to evaluate their macroscopic and microscopic properties, which should be done before conducting any further testing. For the identification of powdered components, microscopic examination of herbal materials is essential. In order to prevent contamination, products should be stored in a clean, hygienic environment. Prior to any testing, it should be ascertained that the herbal material is free from contaminants like foreign substances, heavy metals, and aflatoxins (from moulds) [54]. The quality of the plants or their products is influenced by a number of physical, chemical, and geographical factors that also affect the nature of these materials. In addition, the issue of adulteration is becoming a major concern in terms of the quality of herbal materials [75]. Lack of stringent policies for herbal quality control results in intentional or unintentional adulteration, drug substitutions, spurious drugs, and many other practices that are likely to cause substandard quality of the herbal materials that are marketed and consumed for the purpose of human health benefits. Considering all these factors, we aimed to determine the quality control parameters of the plant, which had not previously been reported for *N. herpeticum*. The macroscopic and microscopic parameters were assessed to form the identification profile of the plant. In order to support this, DNA barcoding analysis using rbcL marker was employed for unambiguous identification of the plant. Further analysis revealed the plant to contain no contaminants, particularly heavy metals and aflatoxins. Thus, it was affirmed that the herbal material used in our study is *N. herpeticum* and is free from any possible contaminants. Further, we aimed to assess the toxicity profile of the plant, as it is frequently consumed by certain ethnic groups as an edible wild plant.

Body weight is governed by several factors, including food intake, levels of growth hormone and sex steroids, neurotransmitters, treatment types, and environmental factors [76]. As in the acute toxicity study, both control and test animals showed increased body weight at day 14 (compared to day 0), which suggests no potential toxicity as represented by the normal growth of the animal. Relative organ weight is a sensitive indicator of chemical-induced alteration in organs, and is therefore, considered a conventional measure for elucidating any toxic effect on the organs, particularly the liver [77]. A single dose of 5000 mg/kg did not produce any significant change in the organs of all the animals, which is further supported by the non-significant results of the biochemical parameters measured. This suggests the extract is non-toxic in nature. The normal ranges of the estimated liver biochemical parameters in adult rats (age 7 to 11 weeks) are 45-84 g/L for total protein, 30-48 g/L for albumin, 12-20 g/L for globulin, 132-312 U/L for ALP, 77-157 U/L for AST, and 22-224 U/L for ALT [73]. In the acute toxicity study, the total protein, albumin, globulin, ALP, AST, and ALT were found to be in the normal ranges for both the control and test groups. Based on these observations, it can be deduced that administration of a single fixed dose (5000 mg/kg) of the aqueous *N. herpeticum* extract failed to induce any chronic hepatotoxicity in rats. This was further supported by the lack of behavioural changes or any significant alterations in body weight gain, relative organ weight, or organ morphology. Because there is no evidence of systemic or target organ toxicity with *N. herpeticum*, this plant can be designated as unclassified under GHS classification based on OECD-420 guidelines [55].

In the repeated 28-day oral toxicity study, female rats exhibited a decrease in body weight gain at day 21 and 28, while male rats showed less weight gain from day 14 onwards. This was consistent with the data showing a reduction in water intake in both the male and female groups. However, food intake demonstrated a different pattern, showing a decrease in intake in females from day 14 onward to day 28. On the other hand, the male group showed a significant reduction in food intake (loss of appetite) at day 21 and day 28. Overall, these initial findings indicated a tendency towards the induction of toxicity in both males and females to a certain extent. Despite this, there were no notable physical abnormalities or behavioural patterns, except for a lethargic response in a few animals, which represented a loss of appetite. These findings are correlated with the increase in biochemical parameters in both the female and male groups to varying degrees, representing the possibility of liver injury. To mention, though these biochemical changes were statistically significant in difference from the control group, some of the limits were within the lower and higher limits prescribed in previous literatures [73]. We believe these changes are due to the difference in methodologies employed, and thereby, the upper limit and lower limit are not in line with the previous report. Being the primary target of toxicity, the liver is susceptible to injury. Liver damage may impact the structural integrity of hepatocytes, causing the release of membrane-bound enzymes (like AST and ALT) and essential enzymes (like ALP). This leads to histological anomalies, including hepatocyte degeneration [78]. Liver histology suggests that the repeated high dose exposure to the extract may potentially cause liver lesions in the animals. Two female animals displayed leukocyte infiltration and sinusoids dilatation, which is consistent with the changes in biochemical parameters. This implies that repeated high dose administration may potentially cause liver injuries.

Natural product research usually employs either screening of isolated compounds or activity-guided isolation. While the former is expensive and tedious, the latter is preferable given its potential integration of dereplication strategies to eliminate the purification of redundant and known compounds [79]. The integration of robust and sophisticated screening systems such as LC-MS as an early dereplication strategy in natural product research has grown significantly in recent years for the characterization and identification of unknown compounds [80]. In contrast to traditional "magic bullet" theory, network pharmacology exploits the potential of multi-targeted effects of drugs by utilising network analysis, redundancy, and connectivity, along with systems biology [81]. LC-MS detected compounds, when subjected to network pharmacology-based prediction of antibacterial potential, identified TNF and IRAK4 as the genes that may serve a significant role in the transmission of bacterial infection. TNF is a potent pro-inflammatory cytokine produced by activated macrophages and lymphocytes that causes a variety of cellular reactions, including apoptosis and gene expressions involved in immune and inflammatory responses [82]. IRAK4 is a key downstream kinase responsible for the activation of transcription factors like NF-κB associated with innate immunity. IRAK4 is responsible for activating innate immunity through the regulation of a signalling cascade involving MyD88 and tumour necrosis factor receptor–associated factor 6 (TRAF6) [83]. Therefore, impairment of IRAK4 and TNF can interrupt major pathways for inflammation and factors controlling the infection. It can be inferred that the compounds present in *N. herpeticum* can modulate infection associated immune mechanisms via targeting TNF and IRAK4 mediated pathways. Thus, the compounds present in the extract can mediate host responses aside from targeting bacterial components to exhibit antibacterial activity.

Consistent with the molecular docking and MD simulation of the compounds with drug-likeness properties against four bacterial targets, HME (ΔG binding energy of -37.7 Kcal/mol and docking scores of -4.75, -7.53, -6.15, and -5 against DNA gyrase subunit b, glucosamine-6-phosphate synthase, DHFR, and PBP, respectively) was found to be the most potent molecule. All other compounds also demonstrated satisfactory scores but showed less binding affinity as compared to HME. On the other hand, the extract was found to be bacteriostatic against Gram-negative bacteria, particularly *E. coli*, in the microplate titer assay. However, there was a contrasting result against *P. aeruginosa* at the same concentration range in the well diffusion method. The variation in the well diffusion method and microplate titer assay against *P. aeruginosa* can be associated with the physicochemical parameters of the compounds. Solubility and diffusion issues may have resulted in the lack of antimicrobial activity observed in the well-diffusion method. It is anticipated that the extracts were not readily soluble in the MHB, making it challenging for them to diffuse or pass through the agar medium (MHA) to exhibit an antimicrobial effect. Supporting this fact, SwissADME analysis revealed the most potent compound, HME, to be moderately soluble in water. Furthermore, unlike the well diffusion method, the compounds can much more easily diffuse into the media as the extract was suspended in the media in the microtiter plate assay [69]. Nevertheless, the presence of complex mixtures in the extract and their interactions are also to be considered [84], as the possibility of synergistic or antagonistic interactions might lead to variation in the activity. However, this study provides preliminary evidence of the plausible bacteriostatic activity of the extract and HME against Gram-negative rather than Gram-positive bacteria. As it is known, Gram-negative bacteria produce higher levels of TNF as compared to Gram-positive bacteria [85], so it is possible that the compounds might produce desired host immunity against Gram-negative bacteria by modulating the host immune system by targeting the TNF and IRAK4 signalling pathways.

Inflammation is the initial symptom of infection and injury, and is divided into acute inflammation, which develops rapidly and lasts for a brief period of time, and chronic inflammation, which develops slowly and lasts for a long period of time. Uncontrolled inflammation is a significant factor in the development of a variety of chronic diseases [86]. To validate the ethnopharmacological claim, a potential compound-disease network to understand the molecular anti-inflammatory mechanisms of *N. herpeticum* was established. Analysis of interactions between 14 compound-associated genes and inflammation-linked genes revealed that the plant constituents may modulate TNF, PTGS2, EGFR, STAT3, PPARG, PTGER4, PPARA, NOS2, TRPV1, and JAK2 associated pathways to induce an anti-inflammatory response. Tumour necrosis factor (TNF), an inflammatory cytokine, is essential for orchestrating the inflammatory immune response via number of signalling pathways [87]. TNF promotes inflammation by activating the NF- κB and mitogen-activated protein kinase (MAPK) signalling pathways via transcriptional upregulation of genes encoding proinflammatory mediators, such as cytokines and chemokines. It can also indirectly trigger inflammation by precipitating cell death to induce proinflammatory gene expression in bystander cells [88]. On the other hand, PTGS2, which is induced by the immune response to inflammation, is responsible for the preponderance of prostaglandins produced during the immune response to inflammation. Similarly, the EGFR–ERK pathway, PPARG, PTGER4, PPARA etc. has also been known to play crucial role in inflammatory conditions [89, 90]. This data warrants the possibility of modulation of diverse inflammation-associated pathways by the compounds present in the extract and thus requires further insights into these potential compound-target interactions in inflammatory conditions. This finding was consistent with the in vitro studies that demonstrated plant extract to possess a good anti-inflammatory effect with IC_{50} values of 98.76, 85.73, and 96.16 μg/ml in protein denaturation, proteinase inhibition, and haemolysis inhibition assays, respectively. In support of this, the extract also showed dose- and time-dependent anti-inflammatory effects in vivo, as shown by a decrease in paw swelling volume.

In this study, the quality control parameters, toxicity profile, and ethnopharmacological claims of antibacterial and anti-inflammatory activity were experimentally validated. However, this research has certain limitations. Firstly, some *N. herpeticum* compounds have been neglected (as they were not identified by the databases), which may introduce bias into our study. However, the representative compounds selected here are known to play a crucial role in the pharmacological effect. Our systematic predictions can partially compensate for this loss. Second, there are still deficiencies in the gene expression profile library of the databases, such as the insufficient coverage of compounds, genes, and cell types. Third, despite having drug-likeness properties, fewer compounds could not be determined for their disease gene interactions due to the unavailability of gene profiles, thereby limiting the extensive network interaction analysis. Fourth, the compounds that do not possess drug-likeness properties may have a modulatory effect on the other compounds, which is not disclosed by this study. Moreover, as multiple genes and signalling pathways are involved in the intricate inflammatory response process, the use of in silico tools including molecular docking and MD simulations considering a general target would be arbitrary in the present form. Thus, we could not perform the in silico-based assay and molecular biology studies. We anticipate that the extract or compounds require in-depth investigation for their anti-inflammatory potential, with particular emphasis on inflammation-associated diseases or conditions rather than the inflammatory response alone.