GENERAL DISCUSSION

Since the beginning of civilization, medicinal plants have been used by mankind for its therapeutic values. The widespread use of herbal remedies and health care preparations have been described in the ancient texts such as the Vedas, the Bible, the Charak and Susruta Samhita. The plant based, traditional medicine systems continues to play an essential role in health care, with about 80% of the world’s inhabitants relying mainly on traditional medicines for their primary health care (Owlabi et al., 2007). In India and China, thousands of tribal communities still use folklore medicinal plants to cure ailments (Waller, 1993; Perumal-Samy and Patricrja, 1996). According to the World Health Organization (WHO) around 21,000 plants have been used for medicinal purpose in the world (Cathrine and Nagarajan, 2011). The plants which have been selected for medical use over thousands of years constitute the most obvious choice for examining the current search for therapeutically effective new drugs (Cathrine and Nagarajan, 2011). It is now considered important to screen medicinal plants either in the form of crude extract or as components isolated from them for promising biological activities (Parekh and Chanda, 2007; Taraphdar et al., 2001). Plants synthesize secondary metabolites as natural pesticides that protect them from herbivores and pathogenic microorganisms (Heldt, 2005).

In India much work has been done on ethnomedicinal plants (Maheshwari et al., 1986; Rai, 1989; Negi et al., 1993) and the Himalayas, in general, have served numerous life saving drug plants (Sinha, 1996; Bandopadhyay and Majumdar, 2002). There is an
ever-increasing demand for plant-based therapeutics in both developing and developed countries (Pandey et al., 2011).

Although quite a few ethno-pharmacological plants are applied against a variety of conditions, there are still numerous plants that have not been cross-tested in diseases apart from the traditional applications (Gridling et al., 2009).

The north-east region of India is rich in plant diversity with about 43% of the total of Indian flora due to its diverse agro-climatic condition, variation of topography, altitudes and rainfalls etc. (Maiti, 2004). Out of 315 families of angiosperms in India, more than 200 are represented in northeast India and this region accounts for nearly 50% of the total number of plant species in India as a whole (Tripathi & Barik, 2003). The region is rich in medicinal plants and many other rare and endangered taxa (Chatterjee et al., 2006). Tripura, a small state of the north-eastern region of India is situated in one of the mega diversity region of the world because of her location in the sub-Himalayan area. The state is rich in biodiversity with vast resource of medicinal plants (Dev, 1983; Das et al., 2009; Roy et al., 2010). However, a very few systematic biological studies have been carried out to explore this vast resource of medicinal plants regarding their medicinal properties.

In respect to the above mentioned scenario, the present study was taken up to carry out specific systematic studies on two medicinal plants of Tripura, viz. *Parkia javanica* and *Evolvulus nummularius*, in respect to antibacterial, anti/pro-oxidative, immune-modulatory, anticancer and antileishmanial properties. The plants were selected on the basis of their ethno-medicinal uses. The tribal people of Tripura use *P. javanica*
extract to cure stomach ache and cholera (Majumder et al., 2009). The Mizo tribals use green portion of the fruit to cure wounds and scabies and also eat fruit or young shoot for curing diarrhoea, dysentery and food poisoning (Bhardwaj and Gakhar, 2005). In Manipur, bark extract is given in diarrhoea and dysentery (Sinha, 1996). *Evolvulus alsinoides* L. is used mainly in traditional medicine of East Asia. The plant is used in Ayurveda as a brain tonic in the treatment of neurodegenerative diseases, asthma and amnesia (Goyal & Singh, 2005). In Indian traditional folk medicine, the whole plant of *E. nummularius* is used as a medicine for hysteria, to cure burns, cuts, wounds and scorpion stings (Jain, 1991). In Nepal, the paste of the plant is used to treat scabies (Manandhar and Manandhar, 2002).

According to the objective number one, to study the antibacterial properties, stem bark and leaves of *Parkia javanica* (*P. javanica*) and whole plant of *Evolvulus nummularius* (*E. nummularius*) were used. Methanol crude extracts as well as solvent fractionated parts of both the plants were tested against six bacterial strains, out of which three were Gram positive bacteria *viz. Micrococcus luteus, Staphylococcus aureus* and *Bacillus subtilis* and three were Gram negative bacteria namely *Pseudomonas aeruginosa, Klebsiella aerogens* and *Escherichia coli*. The activity of plant extracts was determined by using agar cup-plate diffusion method (Kavanagh, 1972) measuring the inhibition zone in mm.

The crude as well as fractionated extracts of both the plants were found more effective against Gram positive strains compared to Gram negative ones. All the Gram positive strains, *Micrococcus luteus, Staphylococcus aureus* and *Bacillus subtilis*, were
sensitive towards the extracts. Out of three Gram negative strains, only *Pseudomonas aeruginosa*, was found to be sensitive. Various workers have already shown that Gram positive bacteria are more susceptible towards plant extracts as compared to Gram negative bacteria (Lin *et al*., 1999; Parekh and Chanda, 2006). These differences may be attributed to the differences in the composition of cell walls (Yao *et al*., 1995). Interestingly, in both the cases maximum activity was observed against *P. aeruginosa*, which was multidrug resistant strain.

All the solvent fractions of *Parkia javanica* plants showed almost similar activities to that of the crude methanol extract. However, methanolic extract showed the maximum activity. Phytochemical analysis (Dinda *et al*., 2009) of the *P. javanica* extract revealed presence of β-sitosterol, ursolic acid (pentacyclic triterpene acid), iridoid glucosides along with other mixture of compounds. Regarding fraction distribution of the compounds, β-sitosterol was found in the lowest polar benzene fraction and iridoid glucosides, ursolic acid in the highly polar n-butanol fraction. All the three groups of compounds are reported to have antimicrobial activities (Gupta *et al*., 1980; Davini *et al*., 1986; Ramesh *et al*., 2001; Shokeen *et al*., 2005). As all the fractions were found to be possess anti bacterial principles, the antibacterial activity of all the fractions may be contributed to principles they possessed. However, the higher activity of the crude methanolic extract could be due to the synergistic effect of all the active principles (Bai, 1990).

Regarding *E. nummularius*, the results of the present study corroborate with the findings on antibacterial activity reported by Pavithra *et al*., (2009). The strains, they
used were *S. aureus, B. subtilis, P. aeruginosa, K. pneumoniae* and *E. coli*. Out of five strains, *E. coli* and *B. subtilis* were reported to be sensitive with MIC values 12.5mg/ml and 3.125 mg/ml respectively. In the present study also, *E. coli* and *B. subtilis* were found sensitive however with higher MIC values, 80 mg/ml and 40 mg/ml respectively. *P. aeruginosa* was found to be resistant to the extract. However, in the present study, *P. aeruginosa* was found sensitive with MIC value, 40mg/ml. The differences observed between the studies may be attributed to the differences existing in plants growing in ecologically different areas and collection of plants of different ages and seasons. Indeed, Lucas and Lewis, (1994) had indicated that some higher plants did contain antibacterial principles at certain stages of their development. Therefore, the differences can logically be explained for the simple reason that plants had not been collected from the same area and at the same time of the plant growth.

Phytochemical analysis of *E. nummularius* extract (Dinda et al., 2007) revealed the presence of β-sitosterol and its glucoside, stigmasterol, d-mannitol, ursolic acid and its isomer oleanolic acid (pentacyclic triterpene acid). Therefore, the antimicrobial activity showed by the *E. nummularius* could be contributed to the antimicrobial principles present in the extract.

Plants being the natural resources of antioxidants, one of objectives was also to assess whether plants under investigation possess any anti-oxidative properties or not. Therefore, according to objective number two, the anti/pro-oxidative properties of the plant extracts were studied in respect to the generation of Reactive Oxygen Species (ROS) and nitric oxide (NO) in macrophage cells. ROS in terms of hydrogen peroxide...
generation was estimated using H$_2$O$_2$ sensitive fluorescent dye, DCFDA and nitric oxide generation was estimated by Griess reagent in macrophage cells in *in vitro* condition.

In connection to ROS generation, *P. javanica* and *E. nummularius* acted differently, being pro-oxidative and anti-oxidative respectively. *P. javanica* treated cells showed approximately three fold higher (p<0.001) ROS generation compared to that of the control, at 1.0µg/ml dose. On the other hand two fold decrease (p<0.001) in ROS generation was observed in *E. nummularius* treated cells at the same dose.

In connection to NO generation, both the plants exhibited very strong pro-oxidative property. *P. javanica* and *E. nummularius* showed about eight fold and nine fold increase in NO production in treated cells after 24 hrs, respectively. However, *P. javanica* could induce the response at ten times lower dose (1.0 µg/ml) compared to that observed for *E. nummularius* (10µg/ml).

Therefore, in respect to ROS and NO generation *P. javanica* was pro-oxidant. On the otherhand, *E. nummularius* remained anti-oxidative in respect to ROS generation and pro-oxidative in respect to NO generation. Several investigators have reported anti- as well as pro-oxidative properties of plant extracts (Desmarchelier *et al.*, 1997; Yen *et al.*, 1997; Perez-Gracia *et al.*, 2001; Tian and Hu, 2005). Reactive Oxygen Species are key actors of non-specific immune defense and the toxic potential of ROS is used by the innate immune defense as a powerful weapon against pathogens (Manda *et al.*, 2009). One of the most beneficial functions of NO is also its implication in host defense against intracellular pathogens (viz., *Salmonella* and *Leishmania*) (Gautam and Jain, 2007). Its derivatives such as per-oxynitrite are also strong bactericidal in nature (Gautam and Jain,
2007). ROS appear to activate and modulate apoptosis when cells are under stress (Benhar, 2002). It is reported that ROS levels are increased in cells exposed to various stress agents, including anticancer drugs (Jabs, 1999) and they promote apoptosis by stimulating pro-apoptotic signaling molecules, such as ASK1, JNK and p38 (Benhar et al., 2001, Davis et al., 2001; Tobiume et al., 2001). p53 induced apoptosis by ROS has been reported (Polyak et al., 1997). Reactive nitrogen intermediates also play a central role in apoptotic cell death (Gautam and Jain, 2007) and the role of NO in tumor cytotoxicity is well documented (Chang, 2001). Therefore, pro-oxidant nature of the extracts, especially in respect to NO generation, may have clinical and therapeutic proposition in tumor cytotoxicity and intracellular pathogen killing.

Various reports on ROS and NO in tumor cytotoxicity and immunomodulation, evoked the idea to examine the anticancer and immunomodulatory activities of the plant extracts (Objective number three).

A tumor is a disease state characterized by a proliferation disorder and an apoptosis obstacle. Inducing apoptosis is an efficient method of treating cancers (Hu & Kavanagh, 2003). A set of investigations was initiated to study the anticancer potential of methanolic extract of Parkia javanica (MEPJ) and methanolic extract of Evolvulus nummularius (MEEN) in vitro against different cancer cell lines (including conventional drug resistant cancer line). Doxorubicin resistant ascetic tumor EAC/Dox murine cancer model was undertaken for studying in vivo response as well as the molecular mechanisms of such effect with MEPJ.
Apoptosis, or programmed cell death, is an essential event that plays an important role in organism development (Hidalgo and Ffrench-Constant, 2003; Vaux and Korsmeyer, 1999) and homeostasis (Kucharczak et al., 2003; Cory et al., 2003; Reed, 2001).

The present study showed that both the extracts were effective in imparting growth inhibition in *in vitro* against various human cancer cell lines (including conventional drug resistant lymphoblastic leukemia) in dose dependent manner.

The growth inhibitory effect of MEPJ was more prominent against K562 and CHO (>70%, p<0.001) than against U937 (~35%, p<0.001) at a dose of 25 μg/ ml. Growth inhibitory effect in response to MEEN was observed to be 54.6% (p< 0.001), 58.59% (p< 0.001) and 16.3% (p<0.001) for K562, U937 and CHO respectively, at a dose of 50 μg/ml.

Interestingly, both MEPJ and MEEN inhibited the proliferation of doxorubicin resistant human lymphoblastic leukaemia CEM/ADR 5000 cells by >94% (p<0.001) at 10 μg/ml and 25 μg/ml dose respectively. Regarding dose, MEPJ exhibited two fold higher response compared to that of MEEN.

Drug resistant cells were more sensitive compared to drug sensitive cancer cells and this is a significant observation since drug resistance is the main problem for cancer chemotherapy and worldwide search for new drug with minimal toxicity is on the way.
To study whether the decrease in cancer cells was due to apoptosis, flow cytometric analysis was done in MEPJ treated cells. Results revealed that MEPJ induced apoptosis in these cancer cells when given in vitro.

In vivo assay was performed in Swiss albino mice and Intra peritoneal (i.p.) route was found to be more effective compared to other routes. Twenty mg/kg of MEPJ administration through i.p. route was observed to give protection of about 99% and increased survivality of EAC/Dox bearing mice. Again, confocal microscopic and flow cytometric analysis confirmed induction of apoptosis in tumour cells.

Therefore, MEPJ was found to be very effective inducer of apoptosis in doxorubicin resistant CEM/ADR 5000 and at a dose of 20mg/kg MEPJ could overcome doxorubicin resistant EAC/Dox ascetic cancer (~99%) by 30 days of post treatment and effectively induced apoptosis of EAC/Dox cells.

The potential mechanism that directs a cell to undergo apoptosis exists in a balance between apoptosis induction factors and apoptosis inhibition factors. A search for a safe agent that enhances the levels of expression of tumor suppressor proteins is a worthwhile but relatively under-explored approach towards cancer therapy.

The present study also revealed that MEPJ enhances expression of pro apoptotic molecules like Bad and Bax on the other hand down regulated expression of anti-apoptotic protein Bcl2 in EAC/Dox cells. Bax, being a Bcl-2 family member, not only promotes apoptosis but also counters the protective effect of survival molecule Bcl-2 (Lee
et al., 2001). In fact, over-expression of Bax, has an effect that is associated with the formation of Bax/Bax homodimers, has been shown to accelerate the cell death of murine FL5.12 cells after interleukin-3 withdrawal (Oltvai et al., 1993).

The intrinsic pathway of apoptosis is tightly controlled at mitochondria by Bcl-2 family proteins. Specifically, these proteins regulate the permeability of the mitochondrial outer membrane and thereby control the release of multiple apoptogenic molecules from the intermembrane space (Danial et al., 2004). Since MEPJ enhanced expression Bad and Bax and on the other hand down regulated expression of Bcl2 in EAC/Dox cells, therefore involvement of mitochondria has been reconfirmed by the Caspase 9 cleavage in the observed death pathway. Finally western blot analysis of EAC/Dox cells derived from mice treated with MEPJ suggested activation of hallmark of apoptosis Caspase 3.

The immunomodulatory study is concerned with stimulation in Ag-presenting ability of LB cells by the plant methanol extracts. The study revealed that both plant extracts could enhance antigen presentation of LB cells to class II restricted 7.13 T cells. Enhancement of antigen presentation to T cell is important immunomodulatory property of any drug/extract. Out of the two extracts, maximum enhancement was observed in E. nummularius treated antigen presenting cells. Methanol extracts of P. javanica was found to induce gradual reversal of immunosuppression as evidenced by induction of lymphoproliferation indicating the immunomodulating property of the extract.
In vivo treatment with MEPJ reversed the suppression of lymphoproliferation in EAC/ Dox bearing mice. This indicated that MEPJ was not toxic for normal cells. Moreover, it may also activated the immune cells besides directly killing the cancer cells.

As mentioned earlier, the phytochemical analysis revealed the presence of ursolic acid (pentacyclic triterpene acid), iridoid glucosides, beta-sitosterol in MEPJ. All the compounds are reported to possess anti-tumor and immunomodulatory properties (Han et al., 1988; Lee et al., 1988; Numata et al., 1990; Huang et al., 1994; Liu, 1995; Es-Saady et al., 1996; Choi et al., 2000; Law, 2000; Plat et al., 2000; Awad et al., 2000; Konoshima et al., 2000; Bouic, 2001; Andersson et al., 2003; Salminen et al., 2008). Induction of apoptosis in cancer by these compounds are also reported. Therefore, the plant reported to possess important anti cancer and immunomodulatory principles was confirmed by its observed anti tumor and immunomodulatory activities in the present study. The results of the study are definitely significant from clinical and therapeutic point of view.

In the absence of any effective vaccine, the only means to treat and control leishmaniasis is affordable medication and medicinal plants hold promise as sources of chemical leads for the development of novel therapeutic agents to fight against leishmaniasis (Rates, 2001; Sharma et al., 2009). The rapid emergence of drug resistance by the treatment of parasites with common chemotherapeutics also warrants the development of new drugs for future therapy.
Antileishmanial activity, as mentioned in objective number four, of methanol extracts of *P. javanica* and *E. nummularius* had been carried out against antimony sensitive AG83 and antimony resistant K39 pro mastigote and amastigote forms of the *L. donovani* parasite. Slight or negligible anti promastigote activity was observed when treated with the extracts but significant anti amastigote activity was found. In general, a drug may act directly against the parasite or indirectly by activating macrophage mitochondrial mechanisms such as ROS and NO production, which has been shown to be the most effective antileishmanial mechanisms. Both ROS and NO are known to be involved in parasite killing in the early stage of leishmanial infection in mice, whereas NO alone is involved in the late phase (Murray and Nathan, 1999). The results of this study indicated involvement of macrophage mitochondria in killing of *Leishmania* parasites. *P. javanica* induced ROS generation while *E. nummularius* reduced ROS generation in MΦs. But the extracts of both the plants induced NO production. Treatment of I-MΦs with ROS inhibitor (NAC) and scavenger of NO (L-NMMA) resulted in inhibition of amastigote killing by the extracts, further strengthening the role of ROS and NO in killing. Cytotoxicity assay with MTT showed that the actions of the extracts were not toxic to macrophages up to the maximum dose (100µg/ml), used for *in vitro* studies. Cytotoxicity tests with natural products are important because of the interest in alternative therapies and the therapeutic use of medicinal plants.

Therefore, on the basis of the results that have been observed in the present study, it could be mentioned that the plants investigated may have clinical and therapeutic potential to combat diseases including most life threaten disease like cancer and
leishmaniasis. Results also considerably justified the traditional use of these medicinal plants.