ABSTRACT

STUDIES ON BIOACTIVITIES OF PARKIA JAVANICA (LAMK.) MERR. AND EVOLVULUS NUMMULARIUS (L.) L.: TWO MEDICINAL PLANTS OF TRIPURA.

THESIS SUBMITTED FOR THE AWARD OF DEGREE OF DOCTOR OF PHILOSOPHY IN HUMAN PHYSIOLOGY IN PARTIAL FULFILLMENT OF THE RESEARCH REQUIREMENTS

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Abstract

Title of the thesis: STUDIES ON BIOACTIVITIES OF PARKIA JAVANICA (LAMK.) MERR. AND EVOLVULUS NUMMULARIUS (L.) L.: TWO MEDICINAL PLANTS OF TRIPURA.

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 systematic studies, on two medicinal plants of Tripura, viz. Parkia javanica and Evolvulus nummularius, with known ethno-medicinal background. The study was designed to examine the medicinal properties of the above mentioned plants with the following specific objectives-

1. to assess the antibacterial activity
2. to assess the anti/pro-oxidative activity
3. to assess the anticancer and immunomodulatory activities
4. to assess the antileishmanial activity

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. 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. 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).
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.
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 MEEN. Drug resistant cells were more sensitive compared to drug sensitive cancer cells and this is a significant observation since the 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.

The present study, western blot analysis, 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.

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. *In vivo* treatment with MEPJ reversed the suppression of lymphoproliferation in EAC/Dox bearing mice.

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 promastigote 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. 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 are not toxic to macrophages up to the maximum dose (100µg/ml), used for *in vitro* studies. Cytotoxicity tests with natural products were 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.

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