CHAPTER-III

EFFECT OF ACRYLAMIDE AND HYBANTHUS ENNEASPERMUS ACTIVE PRINCIPLE ON MICE KIDNEY AND TESTIS GLUTATHIONE S-TRANSFERASES
EFFECT OF HYBANTHUS ENNEASPERMUS ACTIVE PRINCIPLE ON ACRYLAMIDE TREATED MICE KIDNEY AND TESTIS GLUTATHIONE S-TRANSFERASES

Of all the Earth’s biological diversity, the plant kingdom is the most essential to human welfare and is extensively exploited for countless purposes including food, fuel, fiber, construction, tools, and medicine. Of these multiple uses, medicinal use is of high significance as plants have been by far the most important source of medicine for humankind throughout our evolutionary history. In broad terms, the study of the relationships between plants and people is known as ethnobotany. The American botanist John W. Harshberger coined the term “ethno-botany” in 1895 to describe studies of plants used by primitive and aboriginal people. His 1896 publication, “The Purposes of Ethnobotany”, is generally accepted as a starting point for this field as an academic discipline (Harshberger, 1896). Given the dual focus on plants and people, the ideal ethnobotanist receives training in a diverse array of interdisciplinary fields including anthropology, archeology, botany, chemistry, linguistics, psychology, ecology, pharmacology, law, and often requires the skills of both a diplomat and explorer (Balick and Cox, 1996).

In Europe, plants and their derivatives have been used for medicinal and aromatic purposes for thousands of years (Hardalova, 1997). Medicinal plant use in antiquity is recorded by Theophrastus, Hippocrates, Pliny and Dioscorides. Dioscorides is most famous for recording the medicinal properties of more than 600 plant species in the 1st century A.D. His five-volume work, De Materia Medica, was the first systematic pharmacopoeia, containing objective descriptions of 1000 different medications. De Materia Medica was translated and preserved by the Arabs, and finally translated back into Latin by the 10th century (MacKinney, 1965) and remained the most influential reference of its kind in Europe up to the seventeenth century.

The use of plants as medicine, however, did not only emerge in Europe but rather concurrent discoveries of plants and their medicinal uses have occurred in all corners of the globe throughout our evolutionary history. In China alone at least 5,000 plants are believed to be used medicinally important (Farnsworth and Soejarto, 1991), and according to Shanan and Zhong-ming (1991), the total number of taxa may
exceed to 10,000. A similar situation exists in Nepal, Sri Lanka, Bangladesh, Pakistan and India where highly developed traditional systems of Ayurveda, Siha, and Unani medicine are still practiced. Today, scientists speculate on the number of medicinal plants that have been both used historically throughout our evolution, and the number which remain in use worldwide. Accurate inventories and national pharmacopoeias of useful medicinal plants are lacking in most countries including India. Farnsworth and Soejarto (1991) provided a higher up-to-date speculative estimate of 35-70,000 plant species, based upon combining the recorded medicinal plant use of the aforementioned major centers of traditional plant-based medicine with the widespread use of more than 250 million indigenous peoples of Africa, North and South America, Pacific Islands and Asia.

Historically, ethnobotanical studies have provided a major source of new pharmaceuticals, and scientists continue to agree that efforts to examine the medical potential of the earth's plants, animals and microorganisms. Clearly, it is in the interests of Western societies to help the world's biological resources and indigenous cultures from cultural erosion and loss of cultural identity. Farnsworth and Soejarto (1991) report impressive facts about the scale of the developed world's dependence upon medicinal plants and the monetary and social values. The estimate that ¼th of all prescription drugs dispensed in the United States are likely to contain one or more ingredients from higher plants is now commonly quoted (Farnsworth and Soejarto, 1985; Farnsworth, 1988; World Resource Institute, 1993). The global monetary value of plant-based pharmaceuticals in the Organization for Economic Cooperation and Development (OECD) countries alone was estimated to exceed 500 billion dollars in 2000 (Principe, 1991).

A number of drugs from ethnobotanical leads have provided significant milestones in Western medicine. Today, about 95 plant species are listed as the source of 121 clinically useful prescription drugs derived from higher plants (Farnsworth and Soejarto, 1985), but a far higher number are contained in the great variety of over the counter medicines now widely available. Many of our most valuable plant-derived drugs, such as digitoxin and tubocurarine, used to treat specific conditions remain unsurpassed in their respective fields and cannot be synthesized. The isolation of opium alkaloids such as morphine in the early part of the 19th century also heralded
the beginning of the reductionist approach whereby plant extracts were replaced by pure active compounds isolated from a plant.

In more recent times, the ethnobotanical approach to drug discovery has led to the isolation of novel compounds such as Prostatin from *Homoepanthus nutans* (Forster) Pax, a small shrub whose bark was used by local healers in this country or Somalia to treat the viral disease yellow fever (Cox, 1994). Prostatin was isolated as a result of bioassay-guided fractionation of a bark extract that had given significant results in a US screening program for compounds with anti-HIV activity. It progressed to phase II clinical trials in the US, but its progress now appears to have been arrested in favor of other anti-HIV drugs. Prostatin is an interesting compound, belonging to the group of diterpenes known as phorbols, many of which activate protein kinase C, a group of enzymes that transfer phosphate during signal transduction. This process “switches on” a number of cellular processes including cell proliferation and are tumor promoters. Prostatin retains the property of activating protein kinase C, but does not show any indication of promoting tumor growth (Cardellina, 1993).

Consumers are expressing a greater and greater interest in natural products, fuelled by the growing awareness of the risks of using synthetic products, and the growing concern about environmental degradation and the loss of physical and spiritual contact with the environment (Greenwald, 1998; Principe, 1991). In recent years there has been a great surge of public interest in the use of herbs and plants (Akerele, 1991). Some scientists have viewed this phenomenon as a modern “herbal renaissance” (Lewington, 1993).

In general, substances found in plants that are not identified nutrients but may promote health and prevent diseases are called phytochemicals. The phytochemical, or herbal, supplement market is among the fastest growing. Although an active area of research, it might be useful to take some time to examine some of the claims being made about phytochemicals and their potential health benefits.

It is estimated that there are 2,50,000 to 5,00,000 species of plants on earth. A relatively small percentage (1-10%) of these is used as food by both humans and other animal species. It is possible that even more are used for medicinal purposes. Most of
the molecules are secondary metabolites, of which at least 12,000 have been isolated and the number estimated to be less than 10% of the total. Useful antimicrobial phytochemicals can be divided into several categories of phenolic and polyphenols, quinones, flavones, flavonoids, flavonols, tannins, coumarins, terpenoids, essential oils, alkaloids, lectins and polypeptides.

Hybanthus enneasperinus are encountered in all forest types, since they withstand different climatic and environmental conditions as stated by Wilson & Agnew (1992) and Hacker & Gaines (1997) that the plants could modify their own environment turning them more favorable to other species through positive feed back mechanisms.

A small perennial herb with woody base and numerous diffuse or ascending branches. Leaves subessile, linear to oblanceolate, margins entire or serrate, stipules gland-tipped.

Throughout the tropics and subtropics of India from Uttar Pradesh to Bengal southwards to Tamil Nadu and Kerala. Flowers solitary, axillary, red, or purple; petals unequal, the lowest much larger than the others with an orbicular or obovate limb with a long claw. Fruit a small subglobose capsule. Seeds ovoid, longitudinally striate, yellowish-white. This herb is considered to be extremely beneficial to men.

Anjana Dewanji (1993) indicated that leaf protein extracted from unwanted aquatic plants could be used for food and feed purposes. A large number of phytochemicals belonging to several chemical classes have been shown to have inhibitory effects on all types of microorganisms in vitro (Cowan, 1999). Biologically active compounds present in the medicinal plants have always been of great interest to scientist working in this field. Bandarunayake (2002) studied on mangrove plants and bioactive compound and chemical constituents were identified which are having medicinal values. Rahman et al. (2007) revealed that because of rich content of carbohydrates and proteins in aquatic plants they can be utilized as food and feed. Example, Alternathera philoxeroids, Eichhornia crassipes, etc. Rahman (2000) reported the crude extract of Trapa bispinosa possesses the antimicrobial and cytotoxic activity and they reported few compounds from the extract of T. bispinosa and also discussed about the antibacterial spectra of the compounds. Daniel (1989)

The present study was conducted to determine the effect of *Hybanthus enneaspermus* active principle on acrylamide treated mice kidney and testis.

**Objectives**

1. To isolate and characterize *Hybanthus enneaspermus* leaf active principle.

2. To study the effect of *Hybanthus enneaspermus* active principle on the mice kidney and testis GST activities.

3. To analyse the effect of mixture of acrylamide and active principle of HE on the mice kidney and testis GSTs using substrate specificity and Immunological cross reactivity analysis.

The mice were treated with *Hybanthus enneaspermus* as discussed in “Materials and Methods chapter”. The enzyme assays and protein determinations were performed as discussed in “Materials and Methods” Chapter.
RESULTS

The Hybanthus enneaspermus active principle extract was isolated using soxhlet evaporation followed by HPLC using the C18 reverse phase chromatography column. The HPLC profile was depicted in Figures-25 to 27.

Both figures on elution from the column showed a single peak at retention time of about 2.146 to 2.169a at the wavelength of 240nm. The HPLC isolated fraction as analysis of thin layer chromatography found to contain two compounds with almost identical values.

The TLC isolated fractions on scanning λ maxima absorption peaks at 217nm and 408nm.

This indicate that the compound have an aromatic molecule linked to a alkyl carboxylic link. Therefore the compound isolated from H E was almost pure active principle used for its effect on mice kidney and testis GSTs and GST associated peroxidase enzymes.
Fig - 25. HPLC Profile on the isolation active principle of *Hybanthus enneaspermus* leaf extract

Figure-26: TLC analysis of the ethanolic extract of the purified sample of *H. enneaspermus*
Figure-27: Absorption spectrum of selected molecule of *H. enneaspermus* ethanolic extract

The mice were treated with various concentrations of acrylamide + HE leaf active principle with an interval of 24 hours and GST and Gpx activities were tabulated for kidney in Table-14 & for testis in Table-15.

**Effect of *Hybanthus enneaspermus* on acrylamide treated mice kidney GSTs in 24 hours interval:**

Substrate specificity studies using seven classical substrates CDNB, EPNP, pNPA, pNBC BSP, CHP and H_2O_2 were performed and GST activities were determined.

The mice kidney tissue GST activities were increased from control to acrylamide and HE leaf active principle treatment of 1mg, 2mg, 3mg and 4mg in the fold of 0.94x, using the substrate CDNB (**Fig-28A**), in the fold of 0.82x, using the substrate EPNP (**Fig-28B**), in the fold of 0.92x, using the substrate pNPA (**Fig-28C**), in the fold of 0.96x, using the substrate pNBC (**Fig-28D**), in the fold of 0.88x, using the substrate BSP (**Fig-28E**).

The GPx II levels of mice kidney were increased from control to acrylamide and HE leaf active principle treatment of 1mg, 2mg, 3mg and 4mg with CHP, in the fold of 0.66x (**Fig-29A**), and the GPx I (Se-independent) levels were also increased with H_2O_2 in the fold of 0.48x (**Fig-29B**), when compared to control mice kidney for 24 hours interval.
Table 14: Effect of Hybanthus enneaspermus (24 hours interval) on the levels of GSTs and GPx of acrylamide treated mice kidney with different substrates

<table>
<thead>
<tr>
<th></th>
<th>CDNB</th>
<th>EPNP</th>
<th>pNPA</th>
<th>pNBC</th>
<th>BSP</th>
<th>CHP</th>
<th>H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>18.22±2.0</td>
<td>0.32±0.4</td>
<td>0.040±0.006</td>
<td>0.268±0.03</td>
<td>0.062±0.004</td>
<td>0.002±0.001</td>
<td>0.362±0.025</td>
</tr>
<tr>
<td>HE</td>
<td>18.01±2.0</td>
<td>0.29±0.4</td>
<td>0.038±0.005</td>
<td>0.261±0.03</td>
<td>0.060±0.004</td>
<td>0.002±0.001</td>
<td>0.352±0.023</td>
</tr>
<tr>
<td>1mg AC and HE</td>
<td>19.20±2.0</td>
<td>0.35±0.4</td>
<td>0.041±0.006</td>
<td>0.270±0.03</td>
<td>0.68±0.004</td>
<td>0.003±0.008</td>
<td>0.722±0.048</td>
</tr>
<tr>
<td>2mg AC and HE</td>
<td>28.22±2.0</td>
<td>0.42±0.6</td>
<td>0.050±0.13</td>
<td>0.349±0.04</td>
<td>0.88±0.007</td>
<td>0.009±0.007</td>
<td>0.708±0.042</td>
</tr>
<tr>
<td>3mg AC and HE</td>
<td>38.24±4.6</td>
<td>0.54±0.61</td>
<td>0.62±0.014</td>
<td>0.366±0.07</td>
<td>0.92±0.009</td>
<td>0.010±0.009</td>
<td>0.729±0.046</td>
</tr>
<tr>
<td>4mg AC and HE</td>
<td>42.29±5.0</td>
<td>0.59±0.7</td>
<td>0.74±0.015</td>
<td>0.413±0.09</td>
<td>0.94±0.009</td>
<td>0.011±0.011</td>
<td>0.741±0.049</td>
</tr>
</tbody>
</table>

One unit of enzyme activity is defined as micromoles of GSH conjugate formed/min/mg protein (CDNB, EPNP, pNPA, pNBC and BSP).

One unit is defined as micromoles of NADPH oxidized/min/mg protein (CHP, H$_2$O$_2$).

Values are average of three separate experiments of three samples. Mean ±SD significant (t-test).
FIGURE-28: Effect of acrylamide and Hybanthus enneaspermus active principle (24 hours interval) on levels of GSTs of mice kidney with battery of substrates

(A) CDNB

(B) EPNP
FIGURE-29: Effect of acrylamide and Hybanthus enneaspermus active principle (24 hours interval) on levels of Gpx of mice kidney with CHP and H₂O₂.
Effect of Hybanthus ennaespermus on acrylamide treated mice testis GSTs in 24 hours interval:

Substrate specificity studies using seven classical substrates CDNB, EPNP, pNPA, pNBC, BSP, CHP and H₂O₂ were performed and GST activities were determined.

The mice testis tissue GST activities were increased from control to acrylamide + H E leaf active principle treatment of 1mg, 2mg, 3mg and 4mg in the fold of 0.54x, using the substrate CDNB (Fig-30A), in the fold of 0.62x, using the substrate EPNP (Fig-30B), in the fold of 0.61x, using the substrate pNPA (Fig-30C), in the fold of 0.82x, using the substrate pNBC (Fig-30D), in the fold of 0.45x, using the substrate BSP (Fig-30E).

The GPx II levels of mice testis were increased from control to acrylamide and H E leaf active principle treatment of 1mg, 2mg, 3mg and 4mg with CHP, in the fold of 0.79x (Fig-31A), and the GPx 1 (Se-independent) levels were also increased with H₂O₂ in the fold of 0.71x (Fig-31B), when compared to control mice testis for 24 hours interval.
Table-13: Effect of *Hybanthus enneaspermus* (24 hours interval) on the levels of GSTs and GPx of acrylamide treated mice testis with different substrates

<table>
<thead>
<tr>
<th></th>
<th>CDNB</th>
<th>EPNP</th>
<th>pNPA</th>
<th>pNBC</th>
<th>BSP</th>
<th>CHP</th>
<th>H₂O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>21.14 ± 3.1</td>
<td>0.58±0.50</td>
<td>0.072±0.008</td>
<td>0.428±0.015</td>
<td>0.090±0.006</td>
<td>0.625±0.018</td>
<td>0.303±0.030</td>
</tr>
<tr>
<td>HE</td>
<td>18.09 ±2.8</td>
<td>0.49±0.40</td>
<td>0.061±0.006</td>
<td>0.404±0.010</td>
<td>0.087±0.003</td>
<td>0.609±0.015</td>
<td>0.285±0.026</td>
</tr>
<tr>
<td>1mg AC and HE</td>
<td>33.09±4.6</td>
<td>0.79±0.7</td>
<td>0.999±0.016</td>
<td>0.489±0.018</td>
<td>0.19±0.007</td>
<td>0.768±0.021</td>
<td>0.398±0.039</td>
</tr>
<tr>
<td>2mg AC and HE</td>
<td>45.12±5.4</td>
<td>0.86±0.56</td>
<td>0.118±0.026</td>
<td>0.527±0.24</td>
<td>0.28±0.014</td>
<td>0.709±0.021</td>
<td>0.306±0.039</td>
</tr>
<tr>
<td>3mg AC and HE</td>
<td>46.19±5.46</td>
<td>0.90±0.61</td>
<td>0.126±0.032</td>
<td>0.536±0.34</td>
<td>0.32±0.018</td>
<td>0.692±0.022</td>
<td>0.265±0.031</td>
</tr>
<tr>
<td>4mg AC and HE</td>
<td>49.99±6.0</td>
<td>0.91±0.64</td>
<td>0.134±0.038</td>
<td>0.610±0.4</td>
<td>0.34±0.020</td>
<td>0.642±0.019</td>
<td>0.221±0.028</td>
</tr>
</tbody>
</table>

One unit of enzyme activity is defined as micromoles of GSH conjugate formed/min/mg protein (CDNB, EPNP, pNPA, pNBC and BSP).

One unit is defined as micromoles of NADPH oxidized/min/mg protein (CHP, H₂O₂).

Values are average of three separate experiments of three samples. Mean ±SD significant (t-test).
FIGURE-30: Effect of acrylamide and Hybanthus enneaspermus active principle (24 hours interval) on levels of GSTs of mice testis with battery of substrates

(A) CDNB

(B) EPNP
FIGURE-31: Effect of acrylamide and Hybanthus neeaspermus active principle (24hours interval) on levels of Gpx of mice testis with CHP and \( \text{H}_2\text{O}_2 \)

(A) CHP

(B) \( \text{H}_2\text{O}_2 \)
Discussion

Although plant-based foods have been eaten since the existence of mankind,(1) there are many drivers for modern man's consumption of plant foods. Plant foods have an incredible range of tastes and aromas. The smell of a fresh apple or the taste of a ripe tomato have been entrenched in our minds as very distinctive and specific sensations. The diversity of texture found in plant foods is also something that is quite unique. Plant foods may have a crisp, granular or creamy texture. The combination of texture, aroma and taste often leads to a unique sensation in plant food consumption.

The search for new molecules, nowadays has taken a slightly different route where the science of ethnobotany and ethno-pharmacognasy are being used as guide to lead the chemist towards different sources and classes of compounds. These compounds may derive by primary or rather secondary metabolism of living organisms. The secondary metabolites are chemically and taxonomically extremely diverse compounds with obscure function. An important part of the natural products from plants, biomolecules and secondary metabolites usually exhibits some kind of biological activities. They are widely used in the human therapy, veterinary, agriculture, scientific research and in countless other areas.

It is estimated that there are 2, 50,000 to 5, 00,000 species of plants on earth. A relatively small percentage (1-10%) of these is used as food by both humans and other animal species. It is possible that even more are used for medicinal purposes. Most of the molecules are secondary metabolites, of which at least 12,000 have been isolated and the number estimated to be less than 10% of the total.

Phytochemicals are naturally occurring, biologically active chemical compounds in plants. The prefix "Phyto" is from a Greek word meaning plant. In plants, phytochemicals act as a natural defense system for host plants and provide colour, aroma and flavour. More than 4000 of these compounds have been discovered to date and it is expected that scientists will discover many more. Any one serving of vegetables could provide as many as 100 different phytochemicals. Phytochemicals are protective and
disease-preventing particularly for some forms of cancer and heart diseases. The most important action of these chemicals with respect to human beings is somewhat similar in that they function as antioxidants that react with the free oxygen molecules or free radicals in our bodies. Free radicals can damage the cells of our bodies and must be removed.

The thousands of phytochemicals that have been discovered are grouped based on function and sometimes source. These groupings include the widely studied, flavanoids, phyto-oestrogens, phytosterols and carotenoids. These classes and others can be further divided into subclasses. To increase consumption of the phytochemicals from fruits and vegetables: Keep fruits and vegetables available and in sight so that you can remember to use them. Drink fruit juice, instead of soft drinks and other fruit-based beverages. Add chopped fruit to cereal, porridge, milkshakes, pancakes, muffins, cakes. Use fruits and vegetables as snacks for example carrots, raw pumpkin, sweet peppers. Use dried fruits such as raisins and prunes as tasty sweet treats instead of candy. In place of salt, use fresh herbs such as chive, thyme and garlic to season food.

Finally, many plant products are consumed because of their real and perceived importance for health and wellbeing. It is well established that plant foods have been shown to contain important nutritional compounds. Both macronutrients and micronutrients are found in fruit and vegetables. The importance of plant products in the human diet has led to the five-a-day and the seven-a-day campaigns which are driven by the nutritional importance of plant foods in terms of essential vitamins, essential trace elements, sources of energy and fibre. Herbs and spices together with specific fruit and vegetables have been used to develop the many region-specific cuisines around the world.

The search for new molecules, nowadays has taken a slightly different route where the science of ethnobotany and ethno-pharmocognasy are being used as guide to lead the chemist towards different sources and classes of compounds. These compounds may derive by primary or rather secondary metabolism of living organisms. The secondary metabolites are chemically and taxonomically extremely diverse compounds
with obscure function. An important part of the natural products from plants, biomolecules and secondary metabolites usually exhibits some kind of biological activities. They are widely used in the human therapy, veterinary, agriculture, scientific research and in countless other areas.

In general, substances found in plants that are not identified nutrients but may promote health and prevent diseases are called phytochemicals. The phytochemical, or herbal, supplement market is among the fastest growing. Although an active area of research, it might be useful to take some time to examine some of the claims being made about phytochemicals and their potential health benefits.

The development of higher phytochemical-containing plant foods will require an element of caution. These foods should be considered as novel foods if they exceed the normal levels of phytochemicals, and normal background levels are largely unknown at this time. The development of such foods should follow a process based on proper scientific data where the benefits to the end user are clearly stated and all risks associated with consumption are carefully evaluated. Nutritional profiles should be linked to cultivars and the new products should be controlled by a regulatory process that takes all these points into account. Some research is also being devoted to the postharvest treatment of these novel food products with the aim of retaining the biological activity of the phytochemicals.

There are many other phytochemicals that may have an impact on human health. Every food plant contains many hundreds of chemical compounds. The importance of some of these may yet be undiscovered or analytical methods not yet developed to allow quantification. For example more than 200 chemical compounds have been identified in cabbage.

New rapid diagnostic and molecular techniques will be particularly important for the rapid identification of phytochemicals. They are biologically active compounds that are found in relatively small amounts in plant food. These compounds have been linked to human health by contributing to protection against degenerative diseases.
The risks associated with high consumption levels of these novel plants should be evaluated fully, especially because it is feasible that these novel plant foods could be introduced into developing countries where they may be the only source of food over an extended period of time. There is compelling evidence that the benefits of developing plants with high levels of phytochemicals that contribute to health and well-being will be high. However, the expectations far outweigh the scientific knowledge to support these expectations at present. Much work is still required to properly evaluate the benefits and risks associated with this approach to plant food development.