Chapter I

Efficacy of two potentized homeopathic drugs and Vitamin C (L-Ascorbic acid), fed singly and in combination, in amelioration of p-DAB induced hepatocarcinogenesis in mice at cytogenetical, biochemical and ultrastructural levels.
Introduction
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In view of the intricacies of conducting meaningful research on homeopathy as mentioned before, the job at hand would be to establish first that potentized homeopathic drugs beyond Avogadro's limit can modulate quantifiable traits in controlled animal experimentations. It is only when the efficacy of the drug in modulating quantifiable traits in diseased / abnormal state versus normal healthy state is demonstrated can the question of understanding the mechanisms of action arise. Further it would be desirable if the process of undergoing progressive alterations leading to a particular diseased state is either already known or can be further known through intelligent experiments. Artificial carcinogenesis is one such area; particularly p-DAB induced hepatocarcinogenesis has been extensively investigated by many workers earlier (Daoust and Molnar 1964; Morin 1965; Palekar and Sirsat 1966; Matsumoto et al 1968; Miller 1970; Poineer and Pitot 1970; Lin and Wu 1974; Ogawa et al 1974; Lin et al 1975, Kadlubar 1976; Kitagawa 1979; Miller et al 1979; Ohmi et al 1981; Fujii 1983; Samuels et al 1983; Hathway 1986, Yamamoto 1994; Yan et al 1998; Hirano et al 2000; Caballero et al 2001; Ohnishi et al 2000) to understand various aspects that are involved in the process of events leading from initiation to promotion, growth and development of liver cancer, particularly in mouse and rats. Formation of adducts has been considered to be a major causal factor of DNA damage by carcinogenic aminoazo dyes. In addition to DNA adduct formation oxidative DNA damage has also been believed to play an important role in the carcinogenic process of DAB (Ohnishi et al 2000). Hepatocarcinogenesis in rats, is essentially a two stage process, i.e. the production of hepatic tumors by a short term feeding of 2-methyl DAB and the subsequent administration of phenobarbital (PB) where DAB acts as initiating agent while phenobarbital as promoting agent of carcinogenesis (Marquardt et al 1970, Penano et al 1971, Solt et al 1977, Pitot et al 1978). However, it was also demonstrated that dietary PB had positive carcinogenic effects only when fed with 2-methyl-N,N-dimethyl-4-aminoazobenzene in the rat, but neither of these when fed alone apparently showed equally positive hepatocarcinogenetic effects in both mice and rats (Penano et al 1971, 1973, Kitagawa 1977, 1979).

In fact, cancer in itself is an enigmatic and burning issue particularly because of the great risk human beings are now facing (approximately 1 in every 5 persons) and the helplessness
for the fact that still there is no guaranteed remedy in any mode of treatment for this dreadful
disease. Further, cancer basically emanates from a breakdown of the regulatory mechanisms
that govern normal cell behavior (Cooper 1997) and because cancer results from defects in
fundamental cell regulatory mechanisms under meticulous genetic control, it is a disease that
ultimately has to be understood at the cytogenetical (molecular), biochemical, histological and
ultra-structural levels. In the progression of various stages of tumorigenesis and subsequent
malignancy many important studies on practically all directions and aspects have been
carried out that helped us to understand many molecular mechanisms leading to alterations in
functioning of a multitude of metabolic processes involving many enzymes and proteins (see
toxicological studies have now established that increase of lipid peroxidation, acid and
alkaline phosphatases along with decreasing level of glutathione denote general cytotoxicity
and hepatocellular dysfunction (Plaa et al 1991; Tomkuni 1970; Comporti 1985, Srivastava
and Pandey 1982; Dranazni 1976; Deboyser et al 1989; Bannegge et al 1993). Therefore
attempts have been periodically made either to block the pathways via which the normal cell
transforms into a cancer cell or promote alteration in the reverse direction to antagonize the
carcinogenic activities to help cell maintain normal state of activities One such chemical
agent is Vitamin C (L-ascorbic acid). Vitamin C is very popular for its antioxidant properties
(Bodannes and Chan, 1979; Chen and Chang 1979; Shamberger 1984) Since its discovery in the late 1920s
(see Davis et al 1991) probably no other chemical has ever been as celebrated as ascorbic acid (AA) (Arrigoni and
Tullio 2002). Albert Von Szent Gyorgyi was awarded the Nobel Prize for the discovery of ascorbic acid in 1937 and
its curing ability of scurvy L-ascorbic acid, the active form of Vitamin C, functions in many hydroxylase reactions
based upon the oxidation-reduction properties of the vitamins (Cameron et al 1979). Much later the antioxidant activity of ascorbic acid, which efficiently scavenges toxic free radicals and other reactive oxygen species (ROS) formed in
cell metabolism was discovered (see Moser and Benedich 1990; Machlin and Huni 1994;
Olson 1995; Basu and Dickerson 1996) Actually ROS are associated with several forms of
tissue damage and diseases also with the process of ageing (Sies 1993). Aerobic
organisms have evolved intricate and inter-related process for protection against the effect of
free radicals and derived toxic species, including both enzymatic and non-enzymatic
defenses However, man and other animals cannot synthesize ascorbic acid because the
gene encoding L-gulono lactone oxidase (GuL-Ox), enzyme catalyzing the last step in AA
biosynthesis is not functional any more (see Nishikimi et al 1991, Arrigoni and Tullio 2002)

Antimutagenic effect of AA had earlier been reported in both lower organisms like
bacteria (Shamberger et al 1979, Khudoley et al 1981) and in mammalian cells (Bruce et al
the other hand, tumor enhancing effects of AA have also been reported by several workers in

\[ \text{L-ascorbic acid (Vitamin C)} \]
some mammalian models like guineapig (Banic 1981), rat (Fukushima et al. 1983), mice (Frith et al. 1980) but interestingly enough anti-carcinogenic effects of AA have also been reported by various authors. The mitotic activity of the transplantable tumors e.g. Sarcoma 37, Krebs-2 and Ehrlich carcinoma in ascites form was inhibited after treatment with mixture of Vitamin C and B12 (Poydock et al. 1979). Remirez et al. (1980) have studied the direct effects of AA and dehydro AA in vitro on human lymphocyte proliferation phytohemaglutinin (PHA) and concanavalin (Con A) stimulation. When the cells were exposed to physiologic and high concentrations of ascorbic and dehydroascorbic acid the cells show more dose dependent poor tritiated thymidine incorporation than the controls without the vitamin. Freidel (1979) reported when D-iso-ascorbic acid and betaine hydrate are treated in combination they have a blocking action at certain cell membrane receptor sites. AA has also been shown to preferentially inhibit cancer cells in tissue culture. L-AA has been shown to suppress the growth of bone marrow cells from patients with acute non lymphocyte leukemia (Park et al. 1980). Bishun et al. (1978) studied the effect of Vitamin C on cell proliferation and DNA synthesis using two tumor lines, hcp and KB. Earlier Benade et al. (1969) showed that ascorbate is highly toxic and lethal to Ehrlich ascites carcinoma cells in vitro. Vitamin C also has been shown to prevent carcinogenesis and tumorigenesis through its antioxidant action (Shamberger 1972; Slaga and Bracken 1977; Dunham et al. 1982; Lee et al. 2002). AA has also been shown to reverse ultraviolet light induced cytotoxicity in Chinese hamster embryo cells (Chan and Black 1977). Schlegel et al. (1969) observed that Vitamin C reduces uroepithelial carcinoma in mice. Reddy et al. (1982) observed that the incidence of colon and kidney tumors was lower in rats fed 0.25% or 1% sodium ascorbate and treated with single dose of dimethylhydrazine than in the animals fed the diet without ascorbate. Earlier Migliozzi (1977) observed that in guineapigs which received 0.3mg/kg/day of ascorbic acid complete tumor regression occurred in 55% of the animals. However, interestingly enough the animals which were given 10mg/kg/day AA slowed tumor inhibition but no regression and in animals maintained at 1gm/kg/day, the tumors grew without any sign of retardation. The stimulation of tumor growth by megadoses of Vitamin C may be due to the fact that Vitamin C may reduce immunological response in treated animals (Kumar and Axelrod 1969; Kaiden and Guthy 1972). However, the growth of solid form of Ehrlich ascites tumor has been found to be significantly slower in mice maintained on distilled water supplemented with 1% AA (Tewfik et al. 1977). Gruber et al. (1980) made histological observations of tissue architecture in mice with tumor supplemented with dietary AA. Morphological and ultra-structural alterations have also been reported in Chinese hamster ovary cell grown in AA supplemented media (O’Conner 1977) and in mesenchymal tumors (Migliozzi 1977). In human beings most patients with malignant disease seem to have minimal stores of Vitamin C (Basu et al. 1974). Large doses of ascorbic acid have been reported to have a palliative effect on terminal cancer patients (Cameron and Campbell 1974). Patients treated with ascorbate were found to have mean survival about 300 days longer than that of controls (Cameron and Pauling 1978). Thus it was felt that the effects of AA in p-DAB induced hepatocarcinogenesis in mice could be of great interest.
Chelidonium majus L (Fam Papaveraceae) is commonly known as greater Celandine or swallow-wort and is an important medicinal plant rich in specific alkaloids. This plant is of great interest for its use in the treatment of various diseases in many countries of Europe and West Asia (Colombo and Bosisio 1996). The plant contains, as major secondary metabolites, isoquinoline alkaloids such as sanguanarine, chelidonine, chelerythrine, berberine, coptisine. Other non-alkaloid compounds, for example, flavonoids and phenolic acids, have also been isolated from the aerial parts of the plant Chelidonium majus extracts and purified compounds from the extracts have been known to exhibit interesting antiviral, antitumor, and antimicrobial properties both in vitro and in vivo (Furusawa et al 1970; Lozyuk 1977; Sethi 1981; Horvath et al 1983; Kery et al 1987; Colombo and Bosisio 1996). Reverse transcriptase activity of RNA tumor viruses have also been reported to be inhibited by some alkaloids of Chelidonium majus (Sethi 1981, 1983, 1985; Kery et al 1987; Tan et al 1991) Antitumor effects of C. majus, particularly of the aerial parts, have been noted and used for the treatment of malignant diseases, although the single chelidonine and protopine are reported to be cytotoxic at therapeutic doses (Sokoloff 1968) However, chelidonine, a benzophenanthridine ternary alkaloid, and chelidonine derivative had some anticancer and bacteno-static activity (Zbierska and Kowalewski 1980) A lot of works on the synthetic alkaloid derivative Ukrain have been done which also showed similar effects like Chelidonium extract, (Klenrok et al 1992). Later Vahensieck et al (1995) studied the effect of C. majus herb extract on choleresis in the isolated perfused rat liver and found that the total extract induced choleresis.

Crude alcoholic extracts (mother tincture) of Chelidonium majus and various potencies derived from the mother tincture are routinely used by homeopathic practitioners in the treatment of various diseases of the liver including malignancy (Boericke 1976; Clarke 1978). However, a thorough experimental research on the efficacy of the microdoses (potentized) of Chelidonium have not been made earlier in any mammalian model Roberfroid (1983) reported anticancer efficacy of a homeopathic dilution of phenobarbital 9C by only noting the mortality rates in rats chronically fed with acetylaminofluorene at 0.03% for a period of 21 days.

Similarly Carcinosin, a nosode from carcinoma has been claimed to act favorably and to have the ability to modify suitably in all cases in which either a history of carcinoma can be elicited or symptoms of the disease itself exist (see Boericke 1967). Though clinically extensively used in patients with suspected carcinoma, to our knowledge no systematic research has so far been carried out on the efficacy of this drug often used intermittently in a high potency (200C) and above, along with some other suitable homeopathic drug selected on the basis of totality of symptoms, (e.g. Chelidonium in case of liver ailments showing symptoms of liver tumor or malignancy; see Boericke 1976, Clarke 1978). Thus the ameliorative role, if any, played by the homeopathic drugs, Chelidonium and Carcinosin, singly and in combination, during p-DAB induced hepatocarcinogenesis in mice was a subject of great interest.
Since the transformation of normal to cancerous state is under genetic control (the proto-oncogene being converted into oncogenes, or mutation or inactivation of tumor suppressor genes or apoptotic genes (e.g. p53, bcl 2 etc)), extensive works have already been carried out on the expression of various genes as abnormal protein products (e.g oncoproteins) (see Cooper 1997, Lewin 2001) A tumor suppressor gene p53 encodes a nuclear phosphoprotein which normally exists as a homotetramer and is involved in the control of cell cycle, apoptosis and DNA repair (Vogelstein and Kinzler 1992) which is commonly altered in cancers by both mutational and deletional events. When the mammalian cells are exposed to ultraviolet rays or chemical insult, cells are transiently arrested at the G1 phase due to increased intracellular levels of p53 protein and G2 phases of the cell cycle and driven into apoptosis. p53 proteins arrest cell cycle at G1 check point and allow the cells to repair the DNA damage. If the damage is enormous and cannot be properly repaired, p53 eventually leads them to apoptosis, and thereby halt progression of cells towards carcinogenesis. Hence p53 is also referred to as "guardian of the genome". Specific interactions of the p53 with DNA up-regulates the expression of the p53 target genes like GADD 45 and p21. p21 is induced in p53 dependent G1 arrest in normal human diploid fibroblast following irradiation, resulting in inhibition of cdk 2-cyclin E kinase activity. The cellular response to genotoxic agents triggers a rapid increase in the total p53 levels mainly through prevention of p53 degradation. Degradation of p53 may involve ubiquitin dependent proteolysis, and regulated changes in p53 degradation may increase p53 protein levels very rapidly and massively in response to specific stimuli (Fisher 1994). p53 over-expression indicates the presence of a p53 mutation commonly

p53 also has been suggested to play a potential role in regulating cell cycle events distinct from those incurred by DNA damage, for example it was found to regulate an apparent spindle checkpoint, which ensures the maintenance of chromosomal diploidy (Cross 1995). Further studies have demonstrated the ability of p53 to activate Bax gene, a bcl-2 dimenization partner with preapoptotic activity, transcriptionally (Miyashita and Reed 1995). The ratio of apoptosis supressing genes Bcl-2, Bcl-xl, Mcl-1 to apoptotic promoting genes like Bax, Bcl-xs, Bak and Bad may control the susceptibility of mammalian cells to apoptosis.

Thus from the above brief literature review it becomes evident that although p-DAB induced hepatocarcinogenesis has been extensively studied and the mechanisms of action of different metabolites of the azo-dye and phenobarbital are fairly known, the efficacy of neither Chelidonium nor Carcinosin in their potentized forms has so far been systematically studied for understanding their efficacy in ameliorating different stages in hepatocarcinogenesis in mice. Further the efficacy of the known antioxidant AA in ameliorating or aggravating p-DAB induced hepatocarcinoma in mice either alone or in combination with either of these homeopathic drugs had neither been studied earlier for the cytogenetical, biochemical or ultra-structural protocols used in the present investigation. Correspondingly, very little works have so far been carried out on ultrastructural changes occurring in liver during the progression of p-DAB induced carcinogenesis in mice (Palekar and Sirsat 1966; Daoust and Molnar 1964; Ogawa et al 1974). Similarly, so far the author is aware, the expression of
cancer related genes like p53 and genes for expressing Bax proteins, if any in these mice and modulations in expressions of such specific cancer genes or apoptotic genes by the action of any homeopathic drug, have not so far been studied.

Thus the present investigation is a modest attempt to make an inroad into the domain of homeopathy, by utilizing some state-of-the-art techniques and methodologies making use of some suitable experimental designs from different disciplines and aims at to achieve the ultimate objectives:

1. to demonstrate unequivocally if potentized homeopathic drugs indeed have abilities to modulate favorably cellular and sub-cellular (ultra-structural) functioning, either alone or in combination with another homeopathic drug or with vitamin C, having known antioxidant activity and

2. if they do, to understand at the molecular level, how they do it.