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ANTIOXIDANT ACTIVITY OF TIBETAN PLANT REMEDIES USED FOR CARDIOVASCULAR DISEASE

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ABSTRACT

Antioxidant activity was measured in 14 plant species incorporated in more than 30% of Tibetan medicines used for cardiovascular disease and related symptoms according to indigenous pharmacopoeias. The study was undertaken in order to explore possible dietary/medicinal elements which may contribute to the reportedly low incidence of cardiovascular disease among Tibetan highlanders despite high hematocrit levels and a high saturated fat / low fruit and vegetable diet. Extracts of *Terminalia chebula*, *Syzygium aromaticum*, *Aquilaria agallocha*, *Santalum album* , *Amomum subulatum*, *Justicia adhatoda* and *Myristica fragrans* were strong scavengers of the 1,1 diphenyl-2-picryl-hydrazyl (DPPH) radical ($P<0.05$). Cu^{2+}-catalyzed low-density lipoprotein (LDL) oxidation was measured *in vitro* using thiobarbituric acid reactive substances (TBARS) formation and monitoring change in absorbency at 234 nm from conjugated dienes. The hexane fraction of *S. aromaticum* significantly reduced LDL susceptibility to oxidation (1339.96±7.01 min. lag time, $P<0.05$), more than three times longer than Trolox® (431.02 ±21.19 min.). Results of TBARS (90 min.: $r=0.71$, $P<0.005$; 180 min.: $r=0.74$, $P<0.005$) and DPPH ($r=0.69$, $P<0.05$) assays positively correlated to conjugated dienes formation. Our results suggest that these plants are likely to contribute to the therapeutic effects of traditional drugs used to treat cardiovascular disease.

RÉSUMÉ

Cette étude vise à examiner la contribution de plantes alimentaire et medicinales à la faible incidence des maladies cardiovasculaires observée chez les montagnards tibétains, une population à risque élevée à cause de niveaux d'hématocrite élevés et d'un régime riche en gras saturés et faible en fruits et légumes. Pour ce fair, jài mesuré le pouvoir antioxydant de 14 plantes se trouvant dans plus de 30% des préparations médicinales tibetaines utilisée dans la pharmacopée traditionelle pour traiter des maladies cardiovasculaire (MCV) ou leurs symptômes. Les extraits de *Terminalia chebula*, *Syzygium aromaticum*, *Aquilaria agallocha*, *Santalum album*, *Amomum subulatum*, *Justicia adhatoda* et *Myristica fragrans* montrèrent une capacité supérieure de réduire le radical 1,1 diphenyl-2-picryl-hydrazyl (DPPH) signalant ainsi un pouvoir antioxydant élevé. L'oxydation *in vitro* de la lipoprotéine à faible densité (LFD) fut mesurée par la formation de produits de réaction avec l'acide thiobarbiturique (TBARS) et par changement d'absorbance à 234 nm provoquée par la formation des dièenes conjugués. La fraction soluble dans l'hexane de l'extrait de *S. aromaticum* a prit trois fois plus de temps a réagir que le Trolox®, (1339.96±7.01 contre 431.02 ±21.19 minutes respectivement), signalant ainsi une capacité élevé de protèger la LFD de l'oxydation. Les résultats des analyses TBARS (90 mn: r=0.71, P<0.005; 180 mn: r=0.74, P<0.005) et DPPH (r=0.69, P<0.05) sont fortement corrélés à la formation des dièenes conjugués. L'ensemble des tests réalisé ici suggère la moitié des des espèces testées contribuent aux effets thérapeutiques des préparations médicinales traditionnelles utilisées pour le traitement des MCV par les montagnards tibétains.

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CONTRIBUTIONS OF AUTHORS

The manuscript was written by the first author under the guidance of the second author (thesis supervisor). Experiments were performed by the first author using materials, equipment and facilities supplied by the second author.

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1. INTRODUCTION

Plants have deeply influenced the development of human culture and behaviour, such that deviations from an existing evolutionary plant-human equilibrium result in potentially adverse effects. Organisms which are well adapted to their environment are usually capable of health maintenance over their reproductive lifetimes. However, human manipulation of the environment in modern times can have detrimental health effects, despite technological advances in medicine and disease treatments. The close relationship between plants and humans is still apparent among indigenous cultures, populations who follow a traditional nonindustrial lifestyle in areas occupied for generations. The rich contributions of indigenous knowledge of plants has had considerable impact on western cultures, such as presenting novel strategies for treating disease and alternative methods of resource management. Investigation of medicinal and dietary plants therefore contributes to the elucidation of the dynamic interrelationships between the maintenance of human health and certain features of the biological and sociocultural environment (Bogin, 1997; Johns, 1990).

As the threat of undernutrition and infectious disease diminishes, the incidence of diet-related diseases among susceptible industrialized populations increases. Cancer, diabetes and cardiovascular disease (CVD) all have a dietary basis. Long-term studies show that post-World War II increases in industrialization, sedentary lifestyles, and sugar and fat consumption in Japan, Israel, several African countries, Polynesia, Micronesia and among Native Americans and Inuit have led to a greater incidence of obesity and cardiovascular disease (Bogin, 1997; Hamilton *et al.*, 1988). In the 1990s, cardiovascular disease and cancer were the two leading causes of death in the United States, Canada, Japan and Europe (Bogin, 1997; Hajjar & Nicholson, 1995).

In order to prolong life expectancy and reduce CVD, dietary guidelines recommend lowering saturated mammalian fat with partial replacement by vegetable oils and generously increasing vegetable, legume and fruit intake, sources of essential antioxidants (Health and Welfare Canada, 1990; Gey, 1990, 1986). A high intake of mammalian saturated fats leads invariably to elevated levels of plasma low-density lipoproteins (LDL), increasing the likelihood of attack from endo- or exogenously produced free radicals (Bankson *et al.*,

1993). The atherogenic potential of an LDL particle depends on a common initiating step, the peroxidation of polyunsaturated fatty acids caused by free radicals. Oxidative modification of LDL leads to a series of processes which exacerbate the formation of an atherosclerotic plaque, leading to the full development of atherosclerosis, the most common form of CVD (Steinberg *et al.*, 1989). Endogenous (eg. superoxide dismutase, peroxidase) and exogenous antioxidants (eg. α-tocopherol, ascorbic acid, carotenoids, flavonoids) play important radical-scavenging roles in protecting LDL, as well as other lipids, proteins and DNA, from oxidative damage. A score of cross-cultural epidemiological studies support the assumption that cardioprotective attributes of a vegetable and fruit-rich diet is partly due to antioxidants contained therein (Rimm *et al.*, 1996; Gey *et al.*, 1993, 1987; Gey, 1986).

Although there are clear synergistic interactions among lifestyles, diet and metabolic diseases, there are populations who do not respond to increased fat and sugar consumption in the same manner, or have adapted their dietary behaviour to counteract disease development (Weiss *et al.*, 1984). The low incidence of coronary heart disease in the French, despite a high-fat diet and smoking tendencies, has been attributed in part to the regular consumption of red wine, and the phenolic substances contained therein (Renaud & de Lorgeril, 1992). Rates of coronary artery disease in China and Japan are lower than in the West despite higher rates of cigarette smoking. Antioxidant tea flavonoids are believed to contribute significantly to this since large quantities of green tea are consumed regularly (Stehle *et al.*, 1988). The Maasai of East Africa derive more than 60 % of their traditional diet from animal fat, yet have a low incidence of cardiovascular disease. The presence of cholesterol-reducing saponins, antioxidant phenolics and other bioactive allelochemicals in plants used as tonics, masticants and in those added to meat-based stews were deemed important cardioprotectants (Chapman *et al.*, 1997; Johns *et al.*, 1996, 1994; Lindhorst *et al.*, unpublished). Inuit hunter-gatherers chronically consume large quantities of saturated fats yet are not prone to cardiovascular disease. Prophylactic effects are provided by the presence of marine-oils containing n-3 fatty acids, which generate a favourable anti-atherogenic eicosanoid combination (Booyens & Louwrens, 1986).

Similarly, Tibetan highland nomadic pastoralists have a low incidence of

cardiovascular disease, despite a diet high in saturated mammalian fat and sodium (Fujimoto *et al.*1989). Moreover, fruits and vegetables are rarely consumed, and unlike the Inuit, fish is never eaten (Beall & Goldstein, 1993). Additional sources of oxidative stress and CVD risk factors relate to chronic exposure to high altitudes, such as increased haematocrit (Goubali *et al.*, 1995; Sorlie *et al.*, 1981), secondary hypoxia (de Groot & Littauer, 1989; Yoshikawa *et al.*, 1982), and increased exposure to ionizing radiation (Jensen *et al.*, 1997; Riley, 1994). Although low CVD incidence in the presence of promotive haemological and environmental risk factors has been identified previously (Fujimoto *et al.*, 1989), we know of no studies that has examined possible explanatory variables, particularly dietary influences.

In view of the multifactoral nature of CVD, it is clear that certain ingested phytochemicals, particularly antioxidants, reduce the risk of CVD development. Dietary plants in this particular population is reduced to one staple, barley (*Hordeum vulgare*), along with copious ingestion of brick tea (*Camellia sinensis*) laden with sodium and butter (Goldstein & Beall, 1990). Although never investigated in this population, barley and tea may contribute as cardio-prophylactics since hypocholesterolemic compounds occur in the former (Wursch & Sunyer, 1997; Lupton *et al.*, 1993), and antioxidant flavonoids in the latter (Matsuzaki & Hara, 1985). Our reasons for studying medicinal, as opposed to dietary plants as possible cardioprotective contributors, are four-fold. Firstly, the diet of Tibetan highlanders varies seasonally, with barley contributing more calories in summer and meat in winter (Beall & Goldstein, 1993). The difficulties entailed in analysing complex dietary behaviour over the expanse of a year is beyond the allotted scope of this study. The well-established traditional medical system on the other hand, is constant throughout the region, and offers a better opportunity to establish a causal relationship. Secondly, Tibetans, like several other indigenous cultures, view food and medicine as belonging to a common continuum (Handa, 1998). As a result, foods and spices are commonly incorporated into medicinal recipes, and dietary changes often prescribed to alleviate disease. Indeed, the majority of plants included in the present study are well-known spices. Thirdly, Tibetan medicine is botanically more diverse than the highlander diet, which is basically comprised

of barley and tea. In our pursuit to study the bioactive properties of plants, the medical system simply offers more to work with. Lastly, this study offers the opportunity to explore a medical system which has, until relatively recently, evolved unimpeded due to geographical and political isolation. As such, biomedicine is only now beginning to investigate the efficacy and benefits of Tibetan medicine to human health.

The rationale of the present study is founded on similar populations who exhibit contradictory patterns to those established by biomedical research as guidelines to health management and disease prevention. We propose that antioxidants contained within ingested medicinal botanicals confer some degree of protection to LDL from oxidative damage, ultimately contributing to the low observed incidence of CVD among Tibetan highlanders. The objective of this study is to examine the antioxidant activity of plant ingredients most often incorporated into composite medicines used for the treatment of CVD. A general free-radical scavenging assay determined the species that were subsequently extracted using solvents of increasing polarity, and tested for their ability to protect LDL from copper-induced oxidation. The results contribute to a better understanding of disease development within the cultural context of a poorly studied population and provide a more complete description of the relationship between humans and their plant environment. As well, establishing efficacy of indigenous medicines using biomedical standards serves as an invaluable foundation from which to expand the scope of inquiry in order to evaluate the therapeutic potential of plants in the context for which they are used (Etkin, 1986). This is the first study to explore a dietary/medicinal plant basis for the apparent lack of CVD among Tibetan highlanders. Moreover, the culinary familiarity of the majority of plants included in this study can interest a broader audience. Although some of the plants under study have confirmed antioxidant activity, most to our knowledge have not been investigated for their ability to inhibit human LDL from peroxidation.

2. LITERATURE REVIEW

2.1. THE SCIENCE OF ETHNOPHARMACOLOGY

Ethnopharmacology is the study of the chemistry and therapeutic action of substances used to alleviate disease and maintain health according to a medical system not based on the precepts of western science and biomedicine (Etkin, 1996). As such, most medications are predominantly botanicals and are therefore closely allied to other scientific fields such as phytochemistry, plant taxonomy, clinical applications and medical anthropology. The direction an ethnopharmacologic study can take ranges from purely anthropological, dealing with the cultural constructs of therapeutics devoid of systemic investigations of a plant's bioactive attributes, to purely pharmacological, which removes a medicine from its sociocultural context. Ethnopharmacologic studies should invariably begin with viewing plants as cultural objects of symbolic import, and considering the context of use, mode of preparation and application. Laboratory analysis can then relate pharmacologic activity to physiological processes and to the specific therapeutic intent of the medicine. Applying biomedical standards to indigenous medicines contributes to a better understanding of how phytochemical, pharmacologic, clinical and ethnomedical variables interact to affect health and disease.

Efficacy of an indigenous medicine need not be necessarily biomedically validated since efficacy is a culturally constructed concept. Depending on the point of view of a plant's user or investigator, the efficacy of a drug may be viewed as physical, semiotic, or social. Although biomedicine tends to see the function of plant medicines as being able to produce a desired physical response, the plant's user may see efficacy in decidedly nonphysical terms. In such cases, the usual interpretive tactic is to assert that the bioactive component of the plant is a latent effect that somehow explains the plant's persistence in the pharmacopoeia. Clearly non-western medical systems are based on concepts of disease and health differently than those perceived by western biomedicine. As such, perceptions of efficacy are shaped by both biological and behavioral variables (Etkin, 1986, 1988a, 1988b, 1996).

Central to ethnopharmacology is the indigenous acquisition of medicinal plant

knowledge, which is directly related to disease load. The risk or prevalence of encountered diseases is directly linked to growth in local pharmacopoeias (Johns, 1990). For instance, sickness among agriculturalists is much more prevalent, pervasive and diverse than among hunter-gatherers and nomads. As such, agrarian societies tend to rely on a greater number of medicines than their hunter-gatherers counterparts to withstand the greater disease load (Logan & Dixon, 1994). Moreover, dramatic dietary changes which occur as a society tends towards an agrarian lifestyle results in a diet that is reduced to the favoured use of only two or three staple foods, as with maize in mesoamerica, or in this case, barley in Tibet.

With the loss of dietary breadth, several agricultural societies have favoured the use of spices, of which many are used medicinally (Logan & Dixon, 1994). Since several indigenous cultures gauge a plant's medicinal efficacy on taste, olfaction, appearance and texture (Johns, 1990, 1994; Etkin, 1996), it is understandable that the quest for spices and flavourings contributed to the development of local pharmacopoeias. Spices have therefore predominated among pharmacologic investigations of food and medicinal plants (Etkin, 1986). Consequently, it is not surprising that plants included for analysis in the present study are predominantly spices. Indeed, spices are incorporated in several composite medicines used to treat an array of disorders. The use of these plants have been viewed by some researchers as mechanical and/or symbolic adjuvants, vehicles, or facilitators of other ingredients. Furthermore, since these plants are common in medicines used to treat a wide range of disorders, any activity attributed to the composite medicine is usually presumed to be due to other ingredients (Etkin, 1986). However, *Ayurvedic* medicine, on which Tibetan medicine is largely based, has long understood spices as important therapeutic agents (Handa, 1998) that either support primary ingredients or are primary constituents themselves.

Compound medicines are a fundamental concept of such medical systems, and present certain obstacles for biomedical investigations. The difficulties in ascribing the biological effects to one of many constituents of a single plant, are further confounded when considering constituents of several other plants. This notwithstanding, the merits of compound medicines should be further investigated in light of potential synergistic, additive and/or antagonistic effects which may result as different constituents interact. For instance,

the *Ayurvedic* drug *trijataka* or *trikatu*, made from equal parts *Zingiber officinale*, *Piper longum* and *P. nigrum*, is recommended for the treatment of respiratory disorders, skin diseases, fat metabolism disorders and filariasis. Biochemical investigations concluded that *P. longum* and *P. nigrum* significantly enhanced the bioavailability of bioactive principles in other medicines (Handa, 1998; Atal *et al.*, 1981).

The use of spices as medicines raises the issue of cultural distinctions between food and medicine. Biomedicine increasingly recognizes the importance of diet in health and disease. As such, research of potentially bioactive chemicals in formerly mundane foods have increased dramatically, and terms such as "nutraceuticals", "pharmafoods" and "functional foods" are beginning to be commonplace (Etkin & Johns, 1998). Indian and Tibetan cultures, among several others (Etkin & Ross, 1982; Wilson, 1977), have long identified diet as an important component of health, and as such, prescribe dietary modifications concurrently with medicines. The distinction between food and medicine is therefore blurred, and a plant may be considered a medicine, a food, a medicinal/healthy food or a nutritious medicine (Etkin, 1988b). This encourages ethnopharmocologic studies to use a multicontextual approach to plant use.

The practical applications and contribution of ethnopharmocologic research can be viewed variably. Some cite the importance of plants and natural products research in biomedicine development, the inclusion of natural compounds in biomedical therapeutics, and the contemporary manufacture of synthetic compounds modeled on natural products (Farnsworth, 1993; Phillipson & Anderson, 1989), implying that ethnopharmocologic research can advance the interests of biomedicine. Others confer the importance of ethnopharmocologic research to its potential contributions to improving global health (Akerele, 1987; Oyeneye, 1985). Over 80% of people in developing nations, particularly rural populations, rely on medicinal plants as part of their primary health care where biomedicines are difficult to acquire (Farnsworth, 1993). In the interest of health-concerned agencies and individuals, the use of plants which are efficacious according to indigenous and biomedical standards can be encouraged *in lieu* of comparably effective pharmaceuticals.

2.2. Oxidative Damage, Antioxidant Defenses and their Role in CVD

2.2.1. Nature of free radicals and other pro-oxidants

The evolution and development of an organism in the presence of oxygen is associated with the generation of reactive oxygen species (ROS). During cellular respiration, more than 95% of the oxygen is used in the mitochondria for efficient energy production via oxidative phosphorylation, while roughly 1-3% become ROS. ROS are either radicals, which are molecules containing at least one unpaired electron, or reactive non-radical compounds capable of oxidizing biomolecules (e.g. H_2O_2) (Halliwell & Gutteridge, 1986, Halliwell, 1997). The most physiologically relevant include peroxide radicals (ROO$^{\bullet}$), the nitric oxide radical (NO$^{\bullet}$), singlet oxygen ($^1O_2$), peroxynitrite ($ONOO^-$), the superoxide radical ($O_2^{\bullet -}$), and hydrogen peroxide (H_2O_2). Although $O_2^{\bullet -}$ and H_2O_2 are not particularly chemically reactive, interaction with transition metal ions, particularly iron and copper, produce a very reactive free radical species, the hydroxyl radical (OH$^{\bullet}$), which has an estimated half life of 10^{-9} seconds, and will attack and damage almost any biomolecule (Diplock *et al.*, 1998).

ROS generation varies in the human organism, although the superoxide radical anion appears to play a principle role in initiating reactive intermediates. Superoxide is formed by enzymatic one-electron reduction of oxygen by xanthine oxidase (*EC* 1.2.3.2), NADPH oxidase, or accidental leakage of the mitochondrial electron transport chain (Diplock *et al.*, 1998; Halliwell, 1996, 1997). It is proposed that leaked ROS from the mitochondrial respiratory chain oxidatively damages mitochondrial DNA, which deteriorates through time and is responsible for the aging process (Halliwell, 1997).

ROS, produced by phagocytic cells such as neutrophils, monocytes, eosinophiles and macrophages as killing agents against foreign organisms, also form part of the primary immune defense in organisms. Consequently, several diseases are accompanied by excessive phagocyte activation which damages surrounding tissues because of ROS production. Chronic inflammatory diseases, such as inflammatory bowel disease and rheumatoid arthritis, are induced in part by excess ROS production as monocytes accrue at a site (Halliwell, 1997).

An organism can also be exposed to additional ROS from external sources. Several pro-oxidants are introduced into the diet (e.g. quinones) or are inhaled (e.g. cigarette smoke and ozone), levels of which are increasing as air pollution intensifies (Pryor *et al.*, 1995). Ultraviolet irradiation of the skin or ionizing radiation can cleave H_2O_2 to yield two hydroxyl radical molecules which react with neighbouring biomolecules immediately upon their generation (Diplock *et al.*, 1998; Bankson *et al.*, 1993).

2.2.2. Enzymatic and non-enzymatic antioxidant defense system

An antioxidant is defined as "any substance that, when present in low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate" (Halliwell & Gutteridge, 1989). Therefore, oxidative stress describes a serious imbalance between ROS and antioxidants (Halliwell, 1997). Several strategies exist to protect biological systems from pro-oxidants. Two forms of superoxide dismutase (SOD) (*EC* 1.15.1.1), one cystolic and copper- and zinc-dependent, the other mitochondrial and manganese-dependent, catalyze $O_2^{\bullet-}$ to H_2O_2. The resulting H_2O_2 is removed by the enzyme catalase and glutathione peroxidase (GSH)(*EC* 1.11.1.9), the latter having two subtypes: one selenium-dependent and utilizing H_2O_2 as a substrate, the other selenium-independent, and catalyzes the degradation of organic peroxides, particularly lipid peroxides. Other enzymes mediate antioxidant functions indirectly by restoring endogenous antioxidant levels. The supply of reduced glutathione is replenished from oxidized glutathione by the action of the NADPH-dependent enzyme glutathione reductase (*EC* 1.6.4.1). Glutathione in turn maintains the pool of reduced ascorbic acid (vitamin C). Peroxides, in addition to being removed by glutathione peroxidase, can also be removed to a lesser degree by the conjugating action of glutathione-S-transferase (*EC* 2.5.1.18). These metabolically inactive glutathione conjugates are then excreted. (Diplock *et al.*, 1998; Bankson *et al.*, 1993).

Another defense strategy is to control levels of free iron and copper ions, which can promote the synthesis of $OH^{\bullet}$. Metal-binding proteins bind metal ions tightly, thus preventing lipid peroxidation or DNA damage. Iron, bound by proteins such as transferrin, lactoferrin, myoglobin and ferritin, can be released as a stimulus from certain processes such

as tissue injury. The catalytic iron could then participate in the Fenton and Haber-Weiss-type reactions to cause acceleration of tissue damage. Ceruloplasmin, a plasma protein which binds copper, also functions indirectly as an antioxidant as it oxidizes Fe^{2+} to Fe^{3+}, which encourages iron binding by transferrin (Bankson *et al.*, 1993).

Although endogenous antioxidants form the front line in suppressing ROS, they are not overwhelmingly synthesized. Antioxidant defense systems are induced as a response to oxidative stress, forming a sort of balance between ROS production and antioxidant levels. Consequently, oxidative damage is never completely prevented (Halliwell, 1997).

2.2.3. Nutrient and nonnutrient antioxidants

Several compounds in the human diet possess free-radical scavenging activity and ultimately modify the body's antioxidant defense systems. The most prominent representatives are ascorbic acid (vitamin C), tocopherols (vitamin E), carotenoids and flavonoids. All of these groups, with the exception of ascorbic acid, represent an array of structurally different compounds (Rice-Evans & Miller, 1996; Sies & Stahl, 1995). Synergistic effects of these various dietary compounds may bring about effects which are not necessarily the properties of the individual constituents (Diplock *et al.*, 1998).

Ascorbic acid, considered one of the most powerful, least toxic natural antioxidants, is water soluble and found in high concentrations in several tissues. In aqueous phase, ascorbic acid is able to scavenge the superoxide radical anion, hydrogen peroxide, the hydroxyl radical, and singlet oxygen. Furthermore, ascorbic acid is able to regenerate tocopherol from the tocopheroxyl radical which is formed on inhibition of lipid peroxidation (Rose & Bode, 1993). Without this important synergism, the risk of accelerated lipoprotein peroxidation is increased. This process therefore allows the transport of a radical load from a lipophilic to an aqueous compartment, where it is more easily handled by enzymatic defense systems (Niki, 1987). On the other hand, ascorbic acid may also have pro-oxidant activity if in the presence of free iron or copper ions, which can generate the hydroxyl radical and initiate lipid peroxidation (Otero *et al.*, 1997). The concentration of free transition metals *in vivo* however is very small since they are tightly bound to proteins (Diplock *et al.*,

1998).

The most efficient lipophilic antioxidant is α-tocopherol, found in membranes and lipoproteins (Niki, 1987). Its most important role appears to be the inhibition of lipid peroxidation, by scavenging lipid peroxyl radicals and yielding lipid hydroperoxides and a tocopheroxyl radical. This radical, which is subsequently reduced and recycled by ascorbic acid or glutathione peroxidase, is less reactive than peroxyl radicals towards PUFA, and therefore acts as a chain-breaking antioxidant (Diplock *et al.*, 1998).

Carotenoids, like tocopherols, are lipophilic antioxidants and are present in lipoproteins such as LDL and HDL. Carotenoids are the most efficient naturally occurring quenchers for the $^1O_2$ radical, and are also efficient in scavenging peroxyl radicals (Palozza & Krinsky, 1992). Although it is shown that carotenoids are consumed upon peroxidation of isolated LDL (Esterbauer *et al.*, 1991), their contribution to the antioxidant defense system of LDL is not clear, since there are presently no known regeneration pathways for oxidized carotenoids (Palozza & Krinsky, 1992).

Examples of dietary nonnutrient antioxidants include phenolics, particularly flavonoids, and terpenes. Flavonoids encompass a large class of polyphenolic compounds which usually occur as *O*-glycosides, and contain a number of phenolic hydroxyl groups attached to ring structures which confers antioxidant activity against free radical species such as the peroxyl, hydroxyl and superoxide radical (Rice-Evans & Miller, 1996; Bors *et al.*, 1990). They are able to inhibit non-enzymatic lipid peroxidation and NADPH-induced lipid peroxidation *in vitro* and inhibit LDL oxidation by macrophages (Brandi, 1992). Plant polyphenols have other biological activities including anticarcinogenic, anti-inflammatory, and estrogenic effects, and as inhibitors of cyclo-oxygenase, lipoxygenase and phospholipase A_2 (reviewed in Rice-Evans, 1995).

2.2.3.1. Sources and bioavailability of dietary antioxidants

Several food- and host-related factors influence the bioavailability of antioxidants. A common food-related factor involves the quantity of nutrient intake, where the amount absorbed decreases in proportion to increased amounts in food. Moreover, the food matrix

in which antioxidants are located influences availability. Host-related factors include genetic factors, nutrient status and absorption modifiers. The latter is illustrated in the case of lipids required to ensure efficient absorption of lipophilic vitamins and carotenoids.

Major dietary sources of ascorbic acid are fruits and vegetables, particularly citrus fruits, tomatoes and cruciferous vegetables. The amount of ascorbic acid which is absorbed is inversely proportional to the size of the dose ingested. Intake at levels which exceed levels required to maintain plasma levels at about 10 mg/L results in excretion of the excess ascorbic acid unchanged (Kallner *et al.*, 1979).

Rich dietary sources of α-tocopherol are vegetable oils and associated products, as well as wheat germ, nuts and green leafy vegetables. Plasma levels in man are approximately 22 μmol/L, and larger intakes have reportedly no detrimental effects (Farrell & Bieri, 1975).

Carotenoids occur in fruits and vegetables and vary in their bioavailability depending on several factors such as co-ingestion of fat or fibre or food processing (Erdman *et al.*, 1993). For example, lycopene from tomato juice has been shown to have a low bioavailability compared to cooked tomatoes in oil (Giovannucci *et al.*, 1995).

A variety of flavonoids occur in fruits, vegetables and beverages such as wine and tea. Little information is available on the bioavailability of flavonoids in humans. Animal studies have shown that flavonoids present in foods cannot be absorbed through the small intestine because they are often bound to sugars as glycosides (catechins are the exception). Hydrolysis of glycosides into aglycones by microorganisms in the colon leaves the flavonoids susceptible to degradation, so only a small fraction of liberated aglycones are available for absorption. In any case, flavonoids are present in plasma in high enough concentrations to have a biological effect (Bourne & Rice-Evans, 1999; Hollman, 1997; Manach *et al.*, 1995). Quercetin has been estimated to reach levels up to 1μM in human plasma (Hollman, 1997).

2.3. The Role of Free Radicals and Antioxidants in Atherogenesis

2.3.1. Increased atherogeneity of oxidatively modified low-density lipoprotein

Several studies have shown that a diet high in saturated fats leads to elevated low-density lipoprotein (LDL) levels in serum (Stallones, 1983). LDL carries on average three fourths of the serum total cholesterol. Each LDL particle has a hydrophobic core composed of cholesterol esters and triglycerides, and a hydrophilic coat composed of phospholipids, free cholesterol and a molecule of apolipoprotein B-100. Upon oxidation, the LDL particle will contain newly formed lipids and lipid oxidation products, some of which are sufficiently polar to leave LDL. Other lipid oxidation products however, can react with various amino acid residues of the apolipoprotein and alter its structure and composition. These oxidatively modified LDL have several new biological actions. They are potent chemoattractants to circulating monocytes due to the lysophosphatidylcholine generated upon oxidation, they significantly inhibit the motility of tissue-localized macrophages, they are cytotoxic to cultured fibroblasts and endothelial cells (Steinbrecher *et al.*, 1990), and may also promote platelet aggregation, modulate growth factor production by cultured cells, and modify prostaglandin synthesis (Bankson *et al.*, 1993).

The current hypothesis on the role of oxididatively modified LDL in atherogenesis involves the development of fatty streaks (Steinberg *et al.*, 1989). With high LDL concentration in the serum, the intimal LDL concentration inside the arterial wall is also increased. Once intimal LDL is oxidized, circulating monocytes are recruited, enter the arterial wall, and undergo phenotypic modifications and are converted to macrophages. The oxidized LDL inhibits the motility of the macrophages, preventing them from returning to circulation. Since macrophages are able to oxidize LDL themselves, the magnitude of LDL oxidation is increased dramatically. Macrophage-colony stimulating factor, which is released by endothelial and smooth muscle cells, induces the expression of the scavenger receptor, which binds oxidized LDL particles (Hajjar & Nicholson, 1995). It is important to note that these scavenger receptors do not bind normal LDL. Oxidatively modified LDL is then engulfed by the macrophage, where the LDL contributes to the cell's transformation into a foam cell. These lipid-laden foam cells contribute to the formation of a fatty streak,

which increases in size with the accumulation of cells and lipids. The fatty streak may ultimately become mineralized to form an atherosclerotic plaque (Selwyn *et al.*, 1997).

The oxidation of LDL *in vitro* is initiated with the superoxide anion ($O_2^{\cdot-}$) and propagation is dependent on lipid oxyradicals (ROO$^{\cdot}$). The first biochemical change within LDL involves the peroxidation of polyunsaturated fatty acids (PUFA), which results in the rapid generation of free radicals. *In vivo*, oxidized LDL is rapidly removed from circulation by sinusoidal endothelial cells in the liver, spleen and bone marrow. The degree of LDL oxidation in the serum is usually thought to be insignificant due to the presence of relatively high amounts of endogenous antioxidants. Therefore, LDL oxidation occurs most frequently in areas where antioxidant concentration is low, such as the subendothelial space of the arterial wall (Hajjar & Nicholson, 1995; Bankson *et al.*, 1993).

2.3.2. Antioxidant therapy in preventative cardiology

Because oxidized LDL is implicated in the pathogenesis of atherosclerosis, treatment with antioxidants could conceivably retard the progress or actually regress the atherosclerotic lesion. Supplementation with ascorbic acid, α-tocopherol or β-carotene has been shown *in vitro* and *in vivo* to increase LDL resistance to oxidation (Jialal & Grundy, 1992). Epidemiological studies have also shown a negative correlation between plasma concentrations of these antioxidants and the risk of CVD (Gey *et al.*, 1993; 1987; Riemersma *et al.*, 1991; Gey, 1990; 1986). It has been shown that oxidation of polyunsaturated fatty acids (PUFA) in LDL is preceded by the sequential loss of its endogenous antioxidants such as α-tocopherol and carotenoids (Esterbauer *et al.*, 1991). This is the basis for the relatively recent suggestion that antioxidants should be part of the human diet in the prevention of CVD (Hajjar & Nicholson, 1995).

In addition to conventional nutrient antioxidants, there is currently a considerable amount of interest in flavonoids, phenylpropanoids and phenolic acids of plant foods which may act as antioxidants or as agents of other cardioprotective mechanisms (Rice-Evans & Miller, 1996). A Dutch epidemiological study showed that coronary heart disease in elderly males was inversely correlated with their intake of flavonoids (Hertog *et al.*, 1993). Dietary

polyphenols from tea, including catechins and catechin/gallate esters, quercetin and kaempferol, have been the focus of most nonnutrient antioxidant studies. Epicatechin gallate has been found to be ten times more effective than α-tocopherol in preventing lipid oxidation (Namiki & Osawa, 1986). It is proposed that these flavonoids are ideally located near phospholipid surfaces, scavenging radicals in the aqueous phase (Rice-Evans, 1995). It has also been shown that flavonoids, particularly quercetin, conserve endogenous α-tocopherol in LDL (de Whalley *et al.*, 1990). Moreover, polyphenols possess metal-chelating potential, thereby protecting tissues from iron and copper-induced oxidative damage (Laughton *et al.*, 1991). High concentrations of some flavonoids however are reported to promote oxidation, accelerating hydroxyl radical production and DNA damage *in vitro* (Canada *et al.*, 1990), while others are able to modify LDL through non-oxidative processes, and encourage its uptake by macrophages (Rankin *et al.*, 1993). The specific mode of antioxidant activity of individual phenols can therefore be due to (1) chelating metal ions , (2) scavenging lipid alkoxyl and peroxyl radicals, or (3) by regenerating α-tocopherol through the reduction of the α-tocopheroxyl radical (Rice-Evans, 1995).

2.4. Cardiovascular Disease in Tibetan Highlanders

Most studies investigating the cardiovascular parameters of Tibetans focus on high altitude adaptations. Cardiovascular pathologies are therefore studied as a function of chronic exposure to hypoxia. Dietary consideration only arise in the event of deviations from trends established by other high altitude populations. In contrast to these populations, certain Tibetan populations have a higher incidence of atherosclerosis and hypertension, yet the incidence of mortality rates due to CVD is reportedly low.

2.4.1. Atherosclerosis

In autopsy studies of 385 Tibetan adults in Lhasa, it was found that atherosclerosis in the aorta and its major branches occurred in 81.8% and coronary atherosclerosis occurred in 66.6 % of the cases, which is higher than has been reported at low altitudes in China (Sun, 1986). In Qinghai, in spite of the low incidence of coronary infarction among Tibetans,

autopsies revealed coronary artery disease was common and showed the same incidence as in lowlanders (Ward *et al.*, 1995).

In contrast, studies of other high altitude populations suggest that coronary artery disease and myocardial infarction are uncommon. Epidemiological studies in South America have shown that angina and electrocardiogram (ECG) evidence of myocardial ischemia are less at altitude than at sea-level (Ramos *et al.*, 1967). In the Bhutan Tibetan population, angina was uncommon and ECG evidence of coronary artery disease was minimal (Jackson *et al.*, 1966). Fujimoto *et al.*, (1989) reported a low incidence of ischemic heart disease among Tibetans of the Naimonanyi mountains region.

2.4.2. Hypertension

Hypertension is a known positive CVD risk factor since elevated pressure increases strain on blood vessels, increasing probabilities of endothelial injury, and allowing formation of atherosclerotic plaques. Fujimoto *et al.* (1989) reported the absence of systolic hypertension among Tibetan highlanders as a cardioprotective factor, which is in accordance with other high altitude populations in South America (Baker, 1978), in the Tien Shan or Pamir (Mirrakhimov, 1978), and in ethnic Tibetans living in Bhutan (Jackson *et al.*, 1966), and in Qinghai (Wu *et al.*, 1983, 1979). In contrast, a relatively high incidence of hypertension among indigenous Tibetans was reported. Sun (1986) observed an age-associated increase in blood pressure, and no tendency for blood pressure to decline with altitude. The incidence was greater in the urban population around Lhasa than in rural populations. A high prevalence has also been reported for Tibetans living in the western high altitude region of Szechuan province and in other parts of Tibet (Sun, 1978). Sehgal *et al.* (1968) observed an age-associated increase in blood pressure among Tibetans living in northern India.

The cause of hypertension is not clear. On the plateau, obesity is uncommon and few smoke (although this is changing). Sun (1986) suspects that high sodium and low potassium intake is responsible. Tibetans consume a large amount of salt, up to one kilogram a month, much of it taken in their tea. Yak butter is also added. For the ethnic Tibetans living in

Bhutan and Nepal, their tea does not contain as much salt or butter (Ward *et al.*, 1995). Therefore, the high salt and butter intake may be an important factor in the high incidence of hypertension among Tibetans.

2.5. Sociocultural Considerations in Evaluating CVD Risk Factors among Tibetan Highlanders

2.5.1. Concepts of CVD in Tibetan medicine

Tibetan medicine is a comprehensive and complex system of diagnostic and therapeutic means, based on cultural and religious beliefs. The most famous and fundamental work in Tibetan medical literature is the *rGyud-bzhi* (the Four Tantras or Four Treatises) which is said to contain, in condensed essence form, the entire teachings of Tibetan medicine. This text, brought to Tibet from India in the eighth century and added to over successive centuries, is still used today among Tibetan physicians (Clifford, 1984). The fundamental element of Tibetan medicine is the three part division *rlun* (wind), *mkris-pa* (bile) and *ban kan* (phlegm), comparable to the Chinese two part division, *ying* and *yang*. Health is maintained if there is an equilibrium between the three "humours". Disease therefore occurs as a result of an imbalance. Each humour is reflective of the three body-mind constitutions, each associated with particular health needs and behaviors, dietary and lifestyle factors, and predisposition to certain diseases and symptoms. Each has its own method of treatment in terms of nutrition, behavior, medicine and external treatments (Finckh, 1984; 1982). Of equal importance is the concept that the physiological, anatomical, intellectual and psychic functions of the human body act as a mirror of the macrocosm.

The cause of disease or humour imbalance include seasonal factors, a variety of evil spirits, improper diet, or wrongful behavior. Diagnosis is accomplished by observation, palpation, urinalysis and questioning. Treatment involves diet therapy, behavior modifications and medicines (Steiner, 1987), the latter most often being herbal, although animal and mineral medicines are also noted. In the *rGyud-bzhi*, concepts and cures for diseases are addressed in chapters 60-102, of the third Treatise entitled *The Oral Instruction Tantra*. According to Tibetan medical concepts, cardiovascular disease is caused by mental

depression, irregular meals, interrupted sleep and violent anger. There are seven recognized types of CVD, each caused by an imbalance of a combination of different humors (*wind* heart pain, *wind* fever, *blood* heart pain, *blood* fever etc.), each associated with specific symptoms and forms of treatment (Dorjee & Richards, 1985). Apparent in the Tibetan cultural concept of disease is that healing is effected by restoring the lost equilibrium of the three humours, and as a holistic therapeutic system does not necessarily involve the symptomatic treatment of a particular organ (Finckh, 1982). Hence, medicines and treatments in Tibetan medicine are tailored to individual needs, which is in contrast to biomedical concepts which promote the use of chemically homogenous medicines designed to target specific organs, with little emphasis on individualism. The complex concepts, diagnosis and treatments of disease, if used in their cultural contexts, therefore entail several obstacles and difficulties for the biomedical investigation of indigenous medicines.

2.5.2. Diet and lifestyle of Tibetan highlanders

Nomadic pastoralism developed approximately 9,000-10,000 years ago, about the same time as agriculture. It is not known when the practice first emerged in Tibet, however it is doubtful that large-scale nomadic pastoralism existed prior to the domestication of the wild yak (*Bos grunniens*). Unlike other nomadic traditions (e.g. Iran, Turkey, Afghanistan, Pakistan), nomadic pastoralism continues to flourish in Tibet due to lack of competition. Typically, farmers encroach on nomadic pasture lands, often through government support, driving pastoralists to more marginal land. The extreme high altitude and unpredictable bitter climate of Tibet, however, have effectively precluded agriculture as an economic alternative (Goldstein & Beall, 1990).

For centuries, Tibetan nomads have subsisted by harvesting products from their yak, sheep and goats, directly consuming some, such as yogurt, butter and meat, and trading others for barley and tea. Beall & Goldstein, (1993), in studying nomads in Phala, Tibet, found a strong culturally driven dietary seasonality where much more calories are consumed in winter than in summer. *Tsamba*, roasted barley flour, is the staple of Tibet and provides a median of 51 to 73 % of caloric intake in summer and 30 to 40 % in winter. Animal

products are consumed throughout the year, with dairy products contributing 12 to 19 % of the total daily summer calories and meat contributing 36 to 53 % of the total winter calories. It was proposed that a lower basal metabolic rate (BMR), the minimum metabolic activity required to maintain life, would compensate for the relatively low caloric intake during the summer months. In fact, Beall *et al.* (1996) found no evidence for lower summer BMR to compensate for the low summer caloric intake of Tibetans. Moreover, BMR in both seasons were found to be within the normal range predicted by the World Health Organization (WHO) international equations. It has been suggested that the accumulated body fat during the winter buffers the summer period of low intake. A higher BMR would have indicated increased total energy expenditure, and since physical activity is known to promote health, a protective advantage would have been conferred. The relationship between BMR and temperature is illustrated in certain populations, where BMR in tropical peoples was found to be generally lower (Henry & Rees, 1991), and those of high altitude Andean and Himalayan regions higher than predicted (Gill & Pugh, 1964). In addition to a BMR within normal range, the percent body fat, fat mass and body weight of Tibetans were consistent with those reported by Norgan (1994) for rural non-European men and women. This suggests that obesity among Tibetans is uncommon.

The principle foods of the Tibetan highlander are yak beef, mutton, barley, and tea. The diet is virtually devoid of fruits and vegetables. Yak, sheep and goat furnish most of the meat; fowl and pigs are rarely eaten as few keep them; and fish is never eaten (Beall & Goldstein, 1993).

Brick tea, besides tea leaves, consists of twigs and leaves of other plants all bound tightly under pressure. There are three varieties: red, yellow and lucky head to which yak butter and salt are added. Consumption of tea is enormous; about 20-30 cups of tea are consumed daily (Sehgal *et al.*, 1968). The added salt in the tea is suspected to contribute to hypertension among certain Tibetan populations (Sun, 1986).

2.5.3. Cardioprotective elements in Tibetan diet and medicine

Barley (*H. vulgare*), Tibet's primary staple food, has confirmed hypocholesterolemic

properties (reviewed in McIntosh *et al.*, 1995). Activity is attributed on one hand to the β-glucan component of soluble dietary fiber which increases the meal bolus viscosity and delays absorption (Wursch & Pi-Sunyer, 1997; Lupton *et al.*, 1993; McIntosh *et al.*, 1991), and on the other hand to tocotrienol and α-linolenic acid (18:3n-3) (Burger *et al.*, 1984). Tocotrienol is proposed to influence cholesterol metabolism by inhibiting a rate-limiting enzyme (HMG-CoA reductase) in cholesterol synthesis. As a tocopherol, it is also an antioxidant, along with other components of barley such as flavanols ((+)-catechin, (-)-epicatechin and (+)-gallocatechin), other tocopherols (α, δ and γ) and carotenoids (lutein and zeaxanthine) (Goupy *et al.*, 1999).

Tea and the polyphenols contained therein have well established antioxidant activity (Matsuzaki & Hara, 1985) and consumption is attributed to numerous health benefits (reviewed in Weisburger, 1999 and Lunder, 1992). Epigallocatechin gallate is a powerful antioxidant and the main component (>30% dry weight) of tea leaves. Epidemiological studies have shown that regular tea drinkers had lower risk of heart disease than nonusers when comparing populations with similar risk factors (Ishikawa *et al.*, 1997; Knekt *et al.*, 1996; Hertog *et al.*, 1995). The effect is related to the observation in cross-cultural studies that tea consumption is associated with reduced serum cholesterol concentrations (Kono *et al.*, 1992), as well as reduced LDL susceptibility to oxidation *in vitro* (de Whalley *et al.*, 1990). However, since brick tea is made from low-quality, old leaves, their phenolic concentration may be reduced. Furthermore, brick tea usually includes leaves from other plants, most often those found growing alongside the tea when collected (Simoons, 1991). Brick tea also contains high levels of fluorine (200-300 times higher than green or black tea) which are responsible for the high incidence of dental and skeletal fluorosis among Tibetans living in Daofu County, Sichuan Province (Cao *et al.*, 1996a, 1996b). Since there is little information available on the properties of brick tea compared to green or black tea, it is difficult to assess its affects on human health.

Several medicines are prescribed for cardiovascular and related ailments according to the *rGyud-bzhi*. Appendix 1 lists the compound medicines used to treat various forms of CVD according to Tibetan medical concepts. The best known Tibetan medicine to be

studied using biomedical standards prescribed for cardiovascular disease has been Padma 28, so named because it was 28th on the list of medicinals sold to Padma AG of Switzerland in the early 1960s by the descendants of the Lamaistic doctors who had journeyed from Mongolia to Europe (Fishman, 1994a). Padma 28 significantly increased the walking distance in patients with stable, intermittent claudation (Drabaek *et al.*, 1993; Smulski, 1991; Schräder *et al.*, 1985) by decreasing the oxidative burst response of monocytes and improving fibrinolysis (Winther *et al.*, 1994). The drug also significantly reduced the size of atherosclerotic plaques in the aorta and restored several immune functions in animal experiments, making it a very effective treatment for ischemic heart disease (Gieldanowski *et al.*, 1992; Wójcicki *et al.*, 1988). The drug's effectiveness has been attributed to its radical-scavenging properties. The 22 raw dried plant ingredients have been reported to contain high levels of heparinoids and flavonoids and are considered "a powerful mixed-plant antioxidant source" (Fishman, 1994b). These studies have not only reinforced the importance of antioxidants as an effective method of preventing and treating CVD, but also indicate the importance of continuing research into the efficacy of Tibetan indigenous treatments.

2.6. Dietary and Environmental Considerations for Evaluating CVD Risk Factors among Tibetan Highlanders

2.6.1. Dietary elements as risk factors for CVD

Several studies present strong and consistent support for the hypothesis that diets high in saturated fat contribute to the development of ischemic heart disease (reviewed in Stallones, 1983). The hypothesis states that excessive ingestion of saturated fat leads to elevation of LDL in serum, which may ultimately lead to atherosclerosis. In contrast, polyunsaturated fats have the ability to lower plasma cholesterol levels and have been used in the treatment and prevention of atherosclerosis, and on an experimental basis, to inhibit thrombosis. In fact, gram for gram, saturated fat is twice as effective in raising the plasma cholesterol level as polyunsaturated fat is in lowering it. Saturated fats are derived primarily from animals sources such as meat and dairy products, whereas poly- and monounsaturated

fats are primarily found in plant sources such as fruits, grains and vegetables (reviewed in Goodnight *et al.*, 1982).

Epidemiological studies show that CVD risk decreases with increased fruit and vegetable intake, primarily owing to their nutrient and nonnutrient antioxidants and fiber content (Greenberg & Sporn, 1996). Depending on seasonality, animal products can contribute up to half of calories consumed in Tibetan highlanders. Without the cardioprotective elements that fruits and vegetables confer through regular consumption, a higher than observed incidence of thrombogenic diseases would have been assumed.

Although sodium restriction clearly decreases blood pressure in hypertensives (reviewed in Beilin, 1992), it is unclear whether there is any cardioprotective benefit for normotensives (Fleet, 1995; Alderman & Lamport, 1990). Sodium, added to the 20-30 cups of tea which indigenous Tibetans consume daily (Sehgal *et al.*, 1968), is proposed to account for the prevalence of hypertension among some Tibetan populations (Sun, 1986; 1978). Hypertension was found to be age-related, rather than a function of high altitude. There are however reports of the contrary in other populations (Fujimoto *et al.*, 1989; Sehgal *et al.*, 1968; Jackson *et al.*, 1966). Hypertension is thought to result from an imbalance between vasoconstrictors such as thromboxane (see below) and vasodilators such as nitric oxide. Increased vasoconstriction, which characterizes arterial hypertension, is associated with a greater production of free radicals. Indeed, nitric oxide is inactivated by reactive oxygen species, thereby contributing to endothelium-dependent contraction and increased vascular resistance (reviewed in de Artinano & Gonzalez, 1999; Kitiyakara & Wilcox, 1998). The therapeutic potential of dietary antioxidants is therefore considerable, and has been shown to improve endothelial vasodilation (Taddei *et al.*, 1998). Tea flavonoids have antioxidant and vasodilator effects, and regular consumption was proposed to reduce blood pressure in hypertensives. However, Hodgson *et al.* (1999) found that tea ingestion actually caused larger acute increases in blood pressure compared to caffeine.

2.6.1.1. Blood lipid profile of Tibetan highlanders

In the only known study to have investigated serum fatty acid composition and serum

apolipoprotein levels of Tibetan highlanders, Fujimoto *et al.*(1989) found a low serum cholesterol and serum apolipoprotein B and apoB/apoA-I ratio compared to Japanese controls. Apolipoproteins are important in stabilizing and conferring specificity to the lipoprotein, allowing them to be recognized by specific receptors on cell surfaces. Apolipoprotein B-100 is associated with LDL, whereas apolipoprotein A-I is associated with high density lipoproteins (HDL). Since high HDL levels are associated with health benefits, and high LDL levels with health risks, it is desirable to have a low LDL-HDL ratio in the interest of preventing CVD (Goodnight, 1982).

The serum total lipids in the Tibetan highlanders however showed a thrombogenic pattern: a high proportion of palmitic acid (16:0) and stearic acid (18:0) and a low proportion of linoleic acid (18:2 n-6). Linoleic acid is an essential fatty acid which is a precursor for arachidonic acid (20:4 n-6) and eicosanoids such as prostaglandins and thromboxanes. There is an abundance of information suggesting that prostaglandins are involved in the development of atherosclerosis (Hirsh *et al.*, 1981). Part of the mechanism by which diet, hypertension and other stressors contribute to CVD development is through an imbalance between thromboxane A_2 (TXA_2) which promotes platelet aggregation and vessel constriction, and prostaglandin I_2 (PGI_2) which has the opposite effects (Hirsh *et al.*, 1981). Tibetans however showed a discrepancy between serum total lipids and serum phospholipids (Fujimoto *et al.*, 1989). Serum phospholipid levels of 16:0 were low and 18:2 were high. The fatty acid composition in serum phospholipids is considered important because of its influence on cell membrane structure and ultimately, the function of membrane enzymes (Norm *et al.*, 1982). It has been suggested that in spite of the thrombogenic fatty acid pattern in serum total lipids, the high proportion of unsaturated fatty acids in serum phospholipids indicates a sort of defense mechanism in fatty acid metabolism against the development of atherosclerosis (Fujimoto *et al.*, 1989).

Eicosapentaenoic acid (20:5 n-3) , also a precursor to eicosanoids, was found in low proportions in both the serum total lipids and serum phospholipids of Tibetans, possibly because fish is never eaten (Fujimoto *et al.*, 1989). Eicosapentaenoic acid has been attributed to the low incidence of CVD among Eskimos who regularly consume fish, a food

with a high ratio of 20:5 to 20:4 (Dyerberg *et al.*, 1978; Hansen *et al.* 1994). The anti-atherogenic properties of 20:5 are due to its interference with platelet aggregation and its ability to dramatically reduce serum lipids (Phillipson *et al.*, 1985), as well as suppress TXA_2 production (reviewed in Herold & Kinsella, 1986). Low levels of 20:5 in Tibetans might therefore be considered a disadvantage in guarding against CVD.

In summary, Fujimoto *et al.* (1989) attributes the low incidence of thrombotic disease among Tibetan highlanders to their low levels of serum cholesterol, LDL and LDL-HDL ratio and high levels of linoleic acid in serum levels, which outweigh the positive risk factors such as a high hematocrit and low levels of serum total and phospholipid eicosapentaenoic acid levels. Considering the diet of Tibetan highlanders which is high in mammalian saturated fats and low in unsaturated fats, it is surprising that their cholesterol and LDL levels are not higher than reported. These findings may indeed suggest an inherent modification in fatty acid metabolism, but may also imply the ingestion of hypocholesterolemic agents in the diet.

2.6.2. High altitude as a risk factor for CVD

High altitude is a multiple environmental stressor which includes, besides hypobaric hypoxia, low temperatures, low humidity, and increased solar and cosmic radiation (de Meer *et al.*, 1995; Ward *et al.*, 1995). Several factors related to altitude might be considered positive risk factors for the development of CVD. Polycythemia is a well documented adaptation to high altitude (Quilici & Vergnes, 1978) and epidemiological studies have suggested that it can be an important factor in the etiology of CVD (Carlson *et al.*, 1979). In addition, studies have shown that oxidative stress increases with prolonged exposure to hypoxia (Simon-Schnass, 1992; de Groot &Littauer, 1989; Yoshikawa *et al.*, 1982), and ionizing radiation (Jensen *et al.*, 1997; Riley, 1994) which may lead to increased LDL oxidation. Whether these factors are atherogenic in high altitude populations is not fully understood.

High altitude populations have adapted to better cope with hypoxic conditions by developing a more efficient oxygen transport system, including increased ventilation, increased alveolar-arterial O_2 diffusion capacity, increased hematocrit, increased arterial O_2

saturation, increased vascularity of the tissues and increased ability of the cells to utilize oxygen despite low pO_2 (Ward *et al.*, 1995). Tibetans have lived in the Himalayas for approximately 50 000-100 000 years, at least twice as long as the Quechua have lived in the Andes (20 000-40 000 years), and if genetic adaptation to hypoxia is assumed, Tibetans represent the most evolved population to have adapted to high altitude. As a consequence, Tibetans are biologically distinct from other high altitude populations in several physiological aspects, most notably the near absence of chronic mountain sickness (Ward *et al.*, 1995).

2.6.2.1. Natural acclimatization of Tibetan highlanders to high altitudes

Since Tibetans differ physiologically from other high altitude populations, it is important to indicate the relevance of each adaptive mechanism in relation to high altitude stressors which may be implicated in the development of CVD. While some adaptations may not be associated with CVD development, they are nonetheless discussed to better define the population under study.

2.6.2.1.1. Increased pulmonary ventilation

An increase in ventilation results in a relative rise in alveolar and arterial pO_2 (Hohenhaus *et al.*, 1995; Mortola *et al.*, 1990). When compared to acclimatized lowlanders, some high altitude natives, notably the Andean population, have a lower pulmonary ventilation (Hackett *et al.*, 1984), which was interpreted as an energy-efficient adaptation (Lahiri & Milledge, 1967) since the work of breathing was reduced. Tibetans however, have ventilation similar to that of newcomers acclimatized to high altitude (Zhuang *et al.*, 1993) and actually ventilate more than do Han Chinese long-term residents (Curran *et al.*, 1997).

One control of ventilation is the hypoxic ventilatory response (HVR) mediated primarily by the carotid bodies. This response is necessary to ensure a sufficient pO_2 in alveolar gas and arterial blood (Schoene, 1984). The HVR is blunted in Andean (Lahiri *et al.*, 1969; Severinghaus *et al.*, 1966) and American (Kryger *et al.*, 1978b) highlanders, due perhaps to an increase in the stimulus threshold (Tenney & Ou, 1977). This adaptation

allows a greater ventilatory reserve to achieve a greater work capacity at high altitude (Schoene, 1984). In contrast, Tibetans have a greater HVR than Han Chinese long-term residents (Curran *et al.*, 1997). Hypoventilation and a blunted HVR have been implicated in the development of chronic mountain sickness among high altitude residents (Sun *et al.*, 1990; Huang *et al.*, 1984; Lahiri, 1984; Kryger *et al.*, 1978a). Coincidentally, Tibetans have a very low incidence of chronic mountain sickness when compared to high altitude natives of China (Pei *et al.*, 1989) and the Americas (Winslow & Monge, 1987).

2.6.2.1.2. Increased pulmonary diffusion capacity

An increase in pulmonary diffusion capacity facilitates oxygen delivery in high altitude natives by increasing the relative pO_2. Increased pulmonary diffusion is accounted for by an increase in the size and number of alveoli, which is reflected in the larger chest circumference and lung volume of native highlanders (Beall, 1982). Tibetans had a larger chest circumference, and hence greater resting vital capacities than Han residents, which was also significantly greater than low altitude residents (Sun *et al.*, 1990). The increased lung capacity allows for increased area of lung diffusion which, along with increased blood volume, results in increased lung diffusing capacity which gives the high altitude native a distinct advantage over the newcomer to altitude (Ward *et al.*, 1995). Zhuang *et al.* (1996) found that Tibetans had lower total ventilation and higher arterial CO_2 tensions than the Han during exercise and concluded that they exhibited more efficient pulmonary gas exchange which compensated for the reduced ventilation.

2.6.2.1.3. Increased hemoglobin

A rise in red blood cells and hemoglobin concentration increases the oxygen carrying capacity that compensate for the reduced arterial oxygen saturation caused by reduced pO_2 (Quilici & Vergnes, 1978). Low critical levels of pO_2 due to hypoxia at the tissue level acts as a stimulus for increased erythropoiesis. Polycythemia however, also has detrimental effects. Hematocrits over 50-55% dramatically increases blood viscosity and increases resistance to blood flow in both the pulmonary and systemic circulations. Fujimoto *et al.*

(1989) reported hematocrit levels of 56.7 % for men and 58.4 % for females of a Tibetan high altitude population.

Andean populations have very high hemoglobin concentrations, associated with a much higher incidence of chronic mountain sickness compared to Himalayan populations (Hackett *et al.*, 1984; Huang *et al.*, 1984). Moreover, the disease has been reported to be much more prevalent in Han Chinese long-term high altitude residents than Tibetans (Huang *et al.*, 1984). It is extremely rare for Tibetan highlanders to achieve hemoglobin concentrations above 22 gm/dl, a suggested cut-off point for chronic mountain sickness (Heath & Williams, 1977), indicating that the normal range of Tibetans' hemoglobin concentration is associated with healthy levels of blood viscosity (Beall *et al.*, 1987). The reason being that Tibetans seem to have genetically adapted by having higher arterial oxygen saturation compared to Han newcomers (Niermeyer *et al.*, 1995). Beall *et al.* (1994, 1997) have reported the presence of a major gene influencing percent oxygen saturation of arterial hemoglobin in Tibetan highlanders using quantitative genetic analyses. Therefore, the erythrocytosis of Tibetan highlanders conveys an advantage in oxygen carrying capacity, but without raising the blood viscosity to a level where blood flow and oxygen delivery are decreased (Beall *et al.*, 1987; Beall &Reichsman, 1984).

2.6.2.1.3.1. Iron metabolism at high altitude

Iron is an important element at high altitudes due to the hematological changes which occur and its role as a transition metal able to promote oxidation. The generation of reactive oxygen species requires a catalyst such as iron. Ferrous iron can generate the extremely reactive hydroxy radical ($OH^{\cdot}$) through reactions with hydrogen peroxide (H_2O_2). The hydroxy radical is in turn capable of initiating the lipid peroxidation chain reaction. (Minotti & Aust, 1987; Aust *et al.*, 1985). Under normal physiological conditions, iron is tightly controlled and regulated and is not found free or available in the body. Ferritin stores iron in a relatively inert form until needed for heme synthesis. A disruption in the normal iron homeostasis can release iron from ferritin and render it capable of participating in free-radical reactions leading to oxidative damage.

The increase in red cell mass as a result of the erythropoietic response to the hypoxic conditions of high altitude affects the red cell-iron turnover rate. High altitude natives of Morococha, Peru (4,540 m) were found to have a red cell-iron turnover rate one-third higher than that of sea level subjects. When a sea-level resident is brought to high altitude, the circulating inert iron in the plasma diminishes after 2 hours upon arrival, indicating that there is a time lapse between iron mobility from the depot organ to the labile pool. Iron supply is maintained shortly after, mainly by increasing intestinal absorption of iron, and possibly by increasing the efficiency of its mobilization from depot organs (Reynafarje, 1966). Furthermore, ferritin is apparently independent of hematocrit. Natives of Morococha demonstrated that ferritin levels, which were found to be within the normal range, were fairly evenly distributed over the entire hematocrit range, indicating that body iron stores do not limit erythropoiesis (Winslow & Monge, 1987). Thus, it seems that iron metabolism at high altitude does not affect the concentration of reactive iron.

2.6.2.1.4. Circulatory system adaptations

Elevated pulmonary arterial pressure, caused by an increase in pulmonary vascular resistance, is commonly seen in acclimatized lowlanders at high altitude, high-altitude natives and those exposed to acute hypoxia (Ward *et al.*, 1995). These changes are considered a maladaptive response to high altitude since they result in little improvement in ventilation-perfusion matching, increased workload for the right ventricle, limited cardiac output reserve and possibly right ventricular decompensation and death. Raised pulmonary artery and right ventricular pressure is due to the hypoxic pulmonary pressor response which is vital in neonatal life. Indeed, the pulmonary arteries of children at high altitudes have far greater muscularization than is normal in sea-level residents (Saldana & Arias-Stella, 1963). These changes should therefore be considered as a response to high altitude and not necessarily an adaptation. Most Andean natives and acclimatized lowlanders to high altitude suffer from raised pulmonary artery pressure, continued muscularization of the pulmonary arteries and right ventricular hypertrophy (Recavarren & Arias-Stella, 1964). In contrast, it was found that the pulmonary vascular resistance and arterial pressures of Tibetan

highlanders were within sea-level norms (Groves *et al.*, 1993).

Another circulatory adaptation is an increase in the number of capillaries in tissues. Microscopic studies in skeletal muscles of experimental animals exposed to chronic hypoxia have shown increased capillarity, suggesting that the vascular dimensions of the systemic circulation are different in highlanders (Hudlicka, 1984). Increased capillarity compensates for the increased blood viscosity, and as a result, systemic blood pressure remains essentially unchanged in humans residing at high altitude (Vogel *et al.*, 1967). In high-altitude populations, systolic pressure was reported to be lower, with diastolic pressure remaining unchanged (Heath, 1988). However, no carefully controlled trials of the effect of high altitude on systemic blood pressure have been reported (Ward *et al.*, 1995), nor have any studies been conducted on the anatomy of the microcirculation in high altitude natives (de Meer *et al.*, 1995).

2.6.2.2. Polycythemia and cardiovascular disease

Several epidemiologic studies have found that an elevated hematocrit was associated with increased risk of developing thrombotic vascular diseases (Sorlie *et al.*, 1981; Carlson *et al.*, 1979; Tohgi *et al.*, 1978; Shurtleff, 1970). High hematocrit values have also been found to facilitate atherosclerosis, although the mechanism through which this influence acts remains unclear. Some believe this effect is due to an increase in blood viscosity (Paul *et al.*, 1963) while others attribute it to disturbances in blood flow (Erslev, 1990) or increased platelet adhesion (Karino & Goldsmith, 1984).

Polycythemia is the primary compensatory mechanism for high altitude living (Erslev, 1990). Himalayan populations who have had a much longer evolutionary exposure to hypoxia have a lesser degree of polycythemia than other high altitude populations (Beall *et al.*, 1987; Beall & Reichsman, 1984). However, since blood viscosity increases linearly at low levels, and rises exponentially at levels of 50% and higher (Erslev, 1990) the hematocrit levels reported by Fujimoto *et al.* (1989) should put Tibetan highlanders at an increased risk of developing thrombotic vascular diseases.

2.6.2.3. Oxidative stress due to hypoxia

Hypoxia may be expected to decrease the formation of reactive oxygen species in biological systems due to decreased pO_2. The most immediate danger comes from the generation of ROS upon re-oxygenation of a previously hypoxic tissue, known as reperfusion injury. (Bulkley, 1987). However, Yoshikawa *et al.* (1982) observed an increase in arterial-tissue lipid peroxidation with increasing hypoxia in animal models, suggesting that low levels of oxygen are as likely as high levels to cause lipid peroxidation. Hypoxia can also increase the vulnerability of the cell to oxidative damage due to the O_2 limitations of mitochondrial cytochrome oxidase which impairs cell homeostasis (de Groot & Littauer, 1989).

Considering the level of adaptation of Tibetan highlanders to the hypoxic conditions of high altitude, it is unlikely that they suffer from oxidative stress. No known study has investigated whether high altitude populations are more subject to oxidative stress as a result of their hypoxic environment. As previously mentioned, their oxygen transport system is as efficient in delivering adequate levels of O_2 in hypoxic conditions as their lowlander counterparts are at sea level.

2.6.2.4. Oxidative stress due to increased solar and cosmic radiation

Because the atmosphere is thinner at high altitudes, it is not as efficient in absorbing the sun's rays, particularly those of the near ultraviolet wavelengths, as well as cosmic radiation entering from space. Moreover, reflection of the sun off the snow greatly increases exposure to radiation (Ward *et al.*, 1995). It was found that lipid peroxidation increases upon exposure to cosmic radiation (Dousset *et al.*, 1996) and in erythrocyte membranes upon exposure to UV radiation (Janssen *et al.*, 1993; Kochevar, 1990). Although radiation is a known source of oxidative stress (Riley, 1994), epidemiological correlation studies on the health effects of chronic exposure to low levels of ionizing radiation on high altitude populations have either found no effect (Eckhoff *et al.*, 1974; Craig & Seidman, 1961) or a lower incidence of certain cancers (Hickey *et al.*, 1981; Mason & Miller, 1974). While some have interpreted the negative correlation as a beneficial effect of chronic low level radiation,

others have suggested that the reduced oxygen availability might have stimulated physiological changes that confer low cancer incidence. Jensen *et al.* (1997) found no significant differences in somatic cell mutation frequencies between Tibetan highlanders and lowland populations, suggesting no adverse health effects from chronic low level radiation exposure. Ionizing radiation exposure should therefore not be considered an important risk factor for the development of CVD.

2.7. Synopsis of CVD Risk Assessments in Tibetan Highlanders

Current knowledge of the effect of lifelong residence at high altitude typically comes from studies of particular populations compared to their lowland counterparts. A major problem in interpreting results is to assess whether the characteristics found to differ between the two populations are really due to the high-altitude environment, or due to racial, nutritional or economic factors. Racial factors are usually eliminated by using low-altitude residence of the same ethnic background as controls. However, it is difficult to control for nutritional factors since high-altitude residents may be economically disadvantaged when compared to their low-altitude controls. Although there are few studies in Himalayan populations, this seems to be the case in the Andes, where the effects of poor nutrition and chronic hypoxia are similar on factors such as growth and development. In contrast, high-altitude residents in Ethiopia are at an economic advantage since the region is free from malaria, and the residents better fed (Ward *et al.*, 1995).

Tibetans represent the most adapted population to hypoxic conditions, and although a genetic factor on the percent oxygen saturation of hemoglobin has been identified (Beall *et al.*, 1997), adaptation is generally thought to be due to phenotypic plasticity. Consequentially, Tibetans are considered a biologically distinct population, characterized solely by their adaptive physiology. The apparent lack of CVD among Tibetan highlanders, despite thrombogenic hematological characteristics, may well be a function of high altitude. Clearly there are some health benefits to high-altitude residence. For example, direct exposure to increased solar radiation inhibits the growth of some bacteria due to the ultraviolet irradiation (Ward *et al.*, 1995), and may also explain lower incidences of cancer

than at low-altitude (Mason & Miller, 1974; Hickey *et al.*, 1981). What has stimulated our interest however, is the unique dietary behaviour of Tibetan highlanders, which although quantified in terms of calories consumed (Beall & Goldstein, 1993), has not been further investigated as being a possible prophylactic against disease. In contrast to other populations, like the French or the Maasai, who ingest cardioprotective compounds as part of what would be considered a thrombogenic diet, Tibetan highlanders have the added element of high-altitude residence, which has direct bearing on hematological features, and ultimately affects CVD risk and development. In our attempt to review and assess possible CVD risk factors, dietary and environmental, the primary consideration remains the biological and physiological distinctness of the population under study. The Tibetan highland diet is unique in itself, and if considered independently, would facilitate interpretations concerning CVD risk. However, dietary behaviour has evolved in direct response to the environment, such that reliance on livestock, and not agricultural products for sustenance, has constituted a more practical alternative for living on harsh Tibetan terrain. A virtually unaddressed issue is whether Tibetan highlanders, whose adaptation to high-altitude is well established, are equally adapted to their high saturated fat, low fruit and vegetable diet. Furthermore, would this dietary adaptation be phenotypic or genotypic? In this population, nutrition and high-altitude residency are directly related, such that considerations of CVD risk cannot be assessed in one factor independently of the other. The issue is clearly complex and multi-faceted, requiring further investigation.

The impact of Tibetan medicine on CVD risk in Tibetan highlanders at first seems ambiguous. The frequency at which medicines are ingested, if at all, within this population is unknown. However, we assume that medicines are accessible for the majority of Tibetans, including nomadic pastoralists, and that local physicians are consulted in the event of a distressing symptom, such as heart pain or angina. Moreover, the multicontextual view of certain plants, as both a food and a medicine, has been discussed previously, particularly in the case of spices. It is presumed that food plants such as spices, considered medicinal and part of the local pharmacopoeia, are incorporated into the cuisine of Tibetan highlanders. As such, we view ingested botanicals, whether from a dietary or medicinal source, as

potentially bioactive elements capable of eliciting a physiological response. In this respect, Tibetan medicines becomes relevant to CVD risk assessment when viewed as belonging to a common continuum with food.

Due to the limited scope of the present study, we have narrowed our considerations concerning CVD risk factors to sources of oxidative stress in high-altitude environments, and to the fat and cholesterol composition of the diet. Hypocholesterolemic and antioxidant compounds in the diet which may confer a health advantage have also been reviewed. Clearly, a plethora of other elements exist which need to be carefully considered when assessing disease risk within a defined population. The present study therefore partly concerns itself with the elucidation of an issue which has not only been little studied, but has never been carefully addressed before.

3. STUDY RATIONALE, HYPOTHESES AND OBJECTIVES

3.1. STUDY RATIONALE

The dietary and environmental uniqueness of high altitude Tibetan populations present an interesting opportunity to further investigate the multifactorial causes of CVD. Unlike other populations that display peculiarities with respect to established cardioprotective dietary and lifestyle guidelines, Tibetans have the added environmental stress of hypoxia and other related high altitude stressors. Possible atherogenic factors in Tibetan highlanders include a diet high in saturated fats, high in sodium, low in fruits and vegetables, increased hematocrit, elevated blood pressure, and low serum eicosapentaenoic acid levels. Conversely, possible cardioprotective factors include hypocholesterolemic agents in barley and antioxidant phenolics in tea, low serum cholesterol and apolipoprotein (apo) B levels and a low apo B/apo A ratio. Although information is lacking, researchers are perplexed at the lack of morbidity among a population that is exposed to and display certain thrombogenic characteristics (Fujimoto *et al.*, 1989). These studies have concentrated on the relationship between chronic exposure to hypoxia and CVD, and less so to nutritional factors. It is proposed in this study that ingested botanicals confer a cardioprotective advantage in Tibetan highlanders, an important consideration which to our knowledge has never been explored. In this respect, considering how Tibetans view food and medicine as belonging to a common continuum, plant ingredients most frequently occurring in cardio-therapeutic composite medicines, which are predominantly spices, were examined for antioxidant activity.

The immediate benefits of the conclusions derived from such research are three-fold. Firstly, the confirmed presence of bioactive phytochemicals in food and medicinal plants will contribute to the exploration of the low incidence of CVD among Tibetan highlanders reported by some researchers, and encourage dietary considerations in future studies. Investigation of the therapeutic potential of ingested botanicals among this unique population are lacking, yet are admittedly a very important aspect in assessing risk factors for metabolic disease development.

Secondly, due to geographical and political isolation, Tibet's indigenous medical

system has remained closed to biomedical investigation until relatively recently. The confirmed biomedical efficiency of Tibetan medicine for intermittent claudation (Schräder *et al.*, 1985; Drabaek *et al.*, 1993; Winther *et al.*, 1994) and arthritis (Ryan, 1997; Ryan & Jamling, 1995) encourages further pharmacological investigations of traditional Tibetan medicines. This study investigates the role of antioxidants in medicines prescribed for CVD-related disorders, and attempts to validate their efficiency biomedically. Efficacy is of course not invalidated in the event of antioxidant inactivity, since different biomechanisms can be affected to reduce susceptibility to CVD.

Thirdly, since the majority of plants investigated in this study are well known spices, the subject matter and results may interest a broader circle. Familiarity with a food increases the likelihood of it being incorporated in one's diet, particularly if it is known to give health benefits. Antioxidant activity is confirmed for several of the plants under study, although none to our knowledge has been investigated for LDL protective effects.

Antioxidant activity was measured using three assays. The first assay measured general free radical scavenging ability using the 1,1 diphenyl-2-picryl-hydrazyl (DPPH) radical, chosen for its ability to accommodate a large sample size (n=15). The ensuing two assays measured different products of lipid peroxidation, which reflected more appropriately *in vivo* conditions relating to atherosclerosis. Different methods available to follow the onset and progression of lipid peroxidation in LDL solutions include measuring the increase of malondialdehyde, lipid peroxides, conjugated dienes, aldehydes, and fluorescent protein or lipids (Esterbauer *et al.*, 1990). None of these methods gives a full and satisfactory picture of lipid peroxidation on its own. Malonaldehyle production and conjugated diene formation were chosen as appropriate parameters based on their time and energy efficiency, ability to accommodate an average number of samples and degree of correlation between the two methods.

The ability of an antioxidant to scavenge the stable free radical 1,1-diphenyl-2-picrylhydrazyl is practical in that it can accommodate a large number of samples in a short period, and is sensitive enough to detect active principles at low concentrations. The DPPH test is a relatively simple, useful and commonly used assay, although it cannot detect

whether oxidized free radical intermediates created from the antioxidants are able to initiate new oxidative chains (Ursini *et al.*, 1994). Because of its odd electron, 2,2-diphenyl-picryl-hydrazyl radical (DPPH) gives a strong absorption band at 517 nm, which vanishes if the electron is paired up. The degree of decolorization is stoichiometric with respect to the number of electrons taken up and therefore, reflective of the antioxidant ability of the crude plant extract.

Plant extracts that showed greatest activity in the DPPH assay were fractionated using organic solvents of increasing polarity, and retested to determine the most active fraction. These fractions were then evaluated for lipid peroxidation inhibition by measuring the increase of malondialdehyde using the thiobarbituric acid reactive substances (TBARS) assay. It is well established that a great variety of aldehydes are formed through the decomposition of lipid hydroperoxides. Approximately 90 % of the TBARS is free malondialdehyde, which produces a red species absorbing at 535 nm (Esterbauer *et al.*, 1987). Reactive malondialdehyde interacts with the functional groups of apolipoprotein B (apo B), and contributes to the altered functional properties of oxidized LDL, ultimately leading to cholesterol loading of macrophages (Esterbauer *et al.*, 1990; Haberland *et al.*, 1982). Criticized for its lack of specificity, the TBARS assay has remained extremely popular with investigators studying lipid peroxidation in biological systems *in vitro*. However, lack of specificity is argued for testing biological tissue samples, and is less a consideration when testing isolated LDL, as is the case in the present study. In addition, direct comparison with other methods of measuring lipid peroxidation have produced comparable results (Slater, 1984). The antioxidant potential of a plant extract is assessed by the amount of detectable thiobarbituric acid reacted malondialdehyde produced over time, where low levels of malondialdehyde signify high antioxidant activity.

The primary products of lipid peroxidation are conjugated lipid hydroperoxides which can be directly measured in the UV spectrum of the aqueous LDL solution (Esterbauer *et al.*, 1988). Continuous monitoring of the oxidation of LDL at 234 nm clearly shows a lag time (period with little or no change in absorption) where little or no lipid peroxidation is occurring. Incubation with antioxidants increases lag times and offers a practical method

for comparing antioxidant efficiency of plant extracts.

3.2. HYPOTHESIS

I) H_a: Crude extract of plants most frequently included in medicines used to treat cardiovascular disease according to Tibetan pharmacopoeias will show significant evidence of antioxidant activity when compared to controls.

H_o: There will be no significant difference in antioxidant activity between crude plant extracts and controls.

II) H_a: Fractions of crude plant extracts most frequently included in medicines used to treat cardiovascular disease according to Tibetan pharmacopoeias demonstrating antioxidant activity will significantly inhibit peroxidation of LDL compared to controls.

H_o: There will be no significant difference in susceptibility of LDL from oxidation between plant fractions and controls.

III) H_a: Correlation between data sets of different experiments will be significant.

H_o: There will be no correlation between methods.

3.3. OBJECTIVES

I) To analyse extracts of plants that occur in 30% or more medicines used to treat cardiovascular disease according to Tibetan pharmacopoeias for general antioxidant activity.

II) To fractionate methanol plant extracts using water, ethyl acetate, hexane and chloroform to aid in the elucidation of the active compound's biochemical properties and identity.

III) To analyse the ability of plant fractions with relatively high antioxidant activity to protect low-density lipoproteins against metal-ion induced oxidation.

IV) To determine the presence of a correlation between the general free radical scavenging ability of plant fractions, levels of released malondialdehyde and appearance of conjugated dienes from peroxidation of low-density lipoprotein incubated with plant fractions with relatively high antioxidant activity.

4. MANUSCRIPT

Antioxidant Activity of Tibetan Plant Remedies used for Cardiovascular Disease

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ABSTRACT

Antioxidant activity was measured in 14 plant species currently incorporated in more than 30% of Tibetan medicines used for cardiovascular disease and related symptoms according to indigenous pharmacopoeias. The study aims at examining possible dietary/medicinal elements which may contribute to the reportedly low incidence of cardiovascular disease among Tibetan highlanders despite high hematocrit levels and a high saturated fat / low fruit and vegetable diet. Extracts of *Terminalia chebula*, *Syzygium aromaticum*, *Aquilaria agallocha*, *Santalum album* , *Amomum subulatum*, *Justicia adhatoda* and *Myristica fragrans* were strong scavengers of the 1,1 diphenyl-2-picryl-hydrazyl radical. Cu^{2+}-catalyzed oxidation of low-density lipoprotein (LDL) was measured *in vitro* using thiobarbituric acid reactive substances (TBARS) formation and monitoring change in absorbency at 234 nm from conjugated dienes. The hexane fraction of *S. aromaticum* significantly reduced LDL susceptibility to oxidation (1339.96±7.01 min. lag time, $P<0.05$), more than three times longer than Trolox® (431.02 ±21.19 min.). Results of TBARS (90 min.: $r=0.71$, $P<0.005$; 180 min.: $r=0.74$, $P<0.005$) and DPPH ($r=0.69$, $P<0.05$) assays positively correlated to conjugated dienes formation. Our results suggest that these plants are likely to contribute to the therapeutic effects of traditional drugs used to treat cardiovascular disease.

4. 1. INTRODUCTION

The nomadic pastoralists of Tibetan highlands have a reportedly low incidence of mortality from CVD despite a high hematocrit caused by residence at high altitude (Fujimoto *et al.*, 1989), a diet high in saturated fats, cholesterol and sodium, and low in fruits and vegetables. We propose that ingested botanicals, from dietary or medicinal sources, contain antioxidants which increase resistance of LDL to oxidative damage, and thus contribute to the low incidence of CVD in Tibetan highlanders. The objective of this study is to assess antioxidant activity and ability to increase resistance of human LDL to copper-mediated oxidation of plant ingredients most frequently incorporated in compound medicines prescribed for CVD and related symptomologies according to Tibetan pharmacopoeias. This is the first known study to investigate dietary/medicinal elements as possible contributors to the reportedly low incidence of CVD among Tibetan highlanders.

4.1.1. Oxidized LDL model of atherosclerosis

The oxidative modification of low-density lipoproteins (LDL) represents one of the major mechanisms implicated in the pathophysiology of atherosclerosis. Oxidized LDL have been shown to promote a number of processes leading to the formation of atherosclerotic plaques in the arterial wall, as well as enhancement of macrophage uptake, cytotoxicity to endothelial cells, immunogenicity, and production of oxysterols, cytokines and growth factors (Steinberg *et al.*, 1989). Elevated levels of LDL can result from a high dietary intake of mammalian saturated fats, and increases the risk of developing several chronic metabolic diseases, notably cardiovascular disease (CVD) (Bankson *et al.*, 1993). Diets high in fat, sugar and salt contribute to a greater incidence of obesity and CVD, one of the two leading causes of death in the United States, Canada, Japan and Europe (Bogin, 1997; Hajjar & Nicholson, 1995). Considerable epidemiological evidence supports the positive effects of a diet high in vegetables, legumes and fruits, which are sources of antioxidant nutrients and flavonoids, in prolonging life and reducing risks of CVD (Gey *et al.*, 1993, 1987; Riemersma *et al.*, 1991; Gey, 1990; 1986). However, some cultures do not respond to increased fat and sugar consumption in the same manner, or have adapted their dietary behaviour to counteract

disease development (Weiss *et al.*, 1984).

The low incidence of coronary heart disease in France, despite a high-fat diet and high smoking incidence, has been attributed in part to the regular consumption of red wine, and the antioxidant phenolic compounds contained therein (Renaud & de Lorgeril, 1992). Low rates of coronary artery disease in China and Japan, which are lower than in the West despite higher rates of cigarette smoking, are ascribed in part to the regular consumption of green tea containing powerful antioxidant flavonoids (Stehle *et al.*, 1988). The Maasai of East Africa derive more than 60% of their traditional diet from animal fat, yet have a low incidence of cardiovascular disease. The presence of hypocholesterolemic and antioxidant phytochemicals in plants used as tonics, masticants and adjuncts to meat-based stews may provide important cardioprotective elements (Chapman *et al.*, 1997; Johns *et al.*, 1994, 1996; Lindhorst *et al.*, unpublished).

4.1.2. Altitude in relation to cardiovascular health

Polycythemia is a well documented adaptation to high altitude (Quilici & Vergnes, 1978). High hematocrit values seem to facilitate atherogenesis through increased blood viscosity (Paul *et al.*, 1963), disturbances in blood flow (Erslev, 1990) and/or increased platelet adhesion (Karino & Goldsmith, 1984). Additional CVD risk factors may include altitude-related sources of oxidative stress such as hypoxia and increased exposure to solar and cosmic radiation. Hypoxia has been observed to increase lipid peroxidation in animal models (Yoshikawa *et al.*, 1982) and renders cells more vulnerable to oxidative damage due to the oxygen limitation of mitochondrial cytochrome oxydase (de Groot & Littauer, 1989). Lipid peroxidation also increases upon exposure to ultraviolet radiation (Janssen *et al.*, 1993; Kochevar, 1990), although epidemiological studies have found no effect in populations exposed to low UV levels (Jensen *et al.*, 1997; Eckoff *et al.*, 1974). Tibetans, however, are considered perhaps the most acclimatized population to high altitude in the world. Some adaptive mechanisms include increased pulmonary ventilation (Curran *et al.*, 1997; Zhuang *et al.*, 1993), increased pulmonary diffusion capacity (Zhuang *et al.*, 1996), and greater arterial oxygen saturation (Beal *et al.*, 1994). Environmental oxidative stress may therefore

be minimal in naturally acclimatized Tibetans.

4.1.3. Diet in relation to cardiovascular health

Possible nutritional risk factors for CVD in Tibetan highlanders include a diet high in sodium, mammalian saturated fats, and very low in fruits and vegetables (Beall & Goldstein, 1993). The principle foods of Tibetan highlanders are yak, sheep and goat meat and dairy products, barley (*Hordeum vulgare*) and brick tea (*Camellia sinensis*) laden with yak butter and salt. Salt, added to the 20-30 cups of tea that are consumed daily (Sehgal *et al.*, 1968), is proposed to account for the prevalence of hypertension among some Tibetan populations (Sun, 1986; 1978). Since diet varies according to season, with barley contributing the majority of calories during the summer (51-73%) and meat during the winter (36-53%), it has been suggested that the accumulation of body fat during the winter buffers the summer period of low intake (Beall *et al*, 1996; Goldstein & Beall, 1990). Hypocholesterolemic soluble fibers in barley (McIntosh *et al.* 1995) and antioxidant flavonoids in tea (Lunder, 1992; Matsuzaki & Hara, 1985) may aid in negating the effects of what would otherwise be considered a thrombogenic diet.

The evolutionary interactions between Tibetans, their environment and diet, is a complex issue when considering the multifactorial nature of CVD. In this respect, we have chosen to focus on traditional Tibetan medicine for four reasons. First, Tibetans, like other indigenous cultures, view food and medicine as belonging to a common continuum (Handa, 1998). As a result, foods and spices are commonly incorporated into medicinal recipes, and dietary changes often prescribed to alleviate disease. Indeed, the majority of plants included in the present study are widely used spices. Second, the Tibet diet varies according to region and season. The well-established traditional medical system on the other hand, is constant throughout the region, and offers a better opportunity to establish a causal relationship. Third, with the exception of barley and tea, the highlander diet is poor in botanical sources. We therefore opted for the medical system which is substantially more botanically diverse. Fourth, this study offers the opportunity to explore a medical system which has, until relatively recently, evolved unimpeded due to geographical and political isolation. As such,

biomedicine is only now beginning to investigate the efficacy and benefits of Tibetan medicine to human health.

4. 2. MATERIALS AND METHODS

4.2.1. Plant selection and acquisition

Plants most frequently incorporated in compound medicines prescribed for cardiovascular symptoms (e.g., heart disease, blood disease, heart fever, chest pain, high blood pressure, and heart tumour) were identified from Tibetan pharmacopoeias (Dash, 1994; Tsarong, 1986; Rinpoche, 1976). Plants occurring in at least 30% of these medicines were selected for analysis (Table 4.2.1.). Approximately 500 g dried plant material was purchased from the Tibetan Medical and Astrological Institute (TMAI; Mens-tsee-khang), Dharamsala, India, stored in plastic bags and kept in a cool dry environment. The TMAI traditionally prepares plant ingredients by sun-drying them, and cleaning and removing foreign material. Plant material was brought back to Montreal for analysis and immediately stored in a -20°C freezer. Plant identification was verified by the author using herbarium and museum specimens housed at the TMAI.

The Tibetan name *gur-gum* is given to both saffron (*C. sativus*) and safflower (*Carthamnus tinctorius*), the former being the preferred form. The latter is usually used by the TMAI due to the costly nature of *C. sativus*. To compare both forms of *gur-gum*, saffron was purchased from Marché Akhavan, 5768 Sherbrooke W, Montreal, Québec.

4.2.2. Preparation of plant extracts

Dried plant material was ground using a Wiley mill with a 850 μm sieve. Samples were extracted with methanol (HPLC grade) using the Soxtec HT extraction system and dried under vacuum. To better identify and characterize the active component, selected methanol crude extracts were partitioned by a series of liquid-liquid extraction between nonmiscible solvents with water. Hexane (HX), chloroform (CL), ethyl acetate (EA) and aqueous (H_2O) fractions were dried under vacuum.

Table 4.2.1. Plant species occurring as ingredients in at least 30% of Tibetan medicines prescribed for cardiovascular disease. Medicinal plant part, harvest origin, frequency of occurrence according to Tibetan pharmacopoeia (Dash, 1994; Tsarong, 1986; Rinpoche, 1973) and extract yield (w/w) are included.

Family, species	English and Tibetan name	Part used	Cultivated / wild	Frequency of occurrence (%)	Extract yield (%)
Acanthaceae *Justicia adhotoda* Nees.	Malabar nut ཨ་ཤ་ཀ་ (Ba śa ka)	Leaves and twigs	W	36.84	15.58
Asteraceae *Carthamus tinctorius* L.	Safflower གུར་གུམ་ (Gur gum)	Flowers	C	63.16*	27.30
Asteraceae *Saussurea lappa* (Decne.) Sch. Bip.	Costus རུ་རྟ་ (Ru rta)	Root	C	47.37	30.20
Asteraceae *Inula racemosa* Hook. f.	Elecampane མ་ནུ་ (Ma-nu)	Root	C	31.58	36.40
Combretaceae *Terminalia chebula* Retz.	Myrobalan ཨ་རུ་ར་ (A ru ra)	Fruit	W	63.16	77.25
Iridaceae *Crocus sativus* L.	Saffron གུར་གུམ་ (Gur gum)	Stigma	C	63.16*	43.40
Myristicaceae *Myristica fragrans* Houtt.	Nutmeg རྫ་ཏི་ (Dza' ti)	Fruit	C	52.63	37.21
Myrtaceae *Syzygium aromaticum* (L.) Merr. & Perry.	Clove ལི་ཤི་ (Li śi)	Flower buds	C	31.58	31.30
Piperaceae *Piper longum* L.	Long pepper པི་པི་ལིང་ (Pi pi ling)	Fruit	C	31.58	31.58
Poaceae *Bambusa arundinaceae* McClure.	Bamboo རྩ་གང་ (Cu gan)	Nodal silica	C	52.63	2.70
Santalaceae *Santalum album* L.	White sandalwood ཙན་དན་དཀར་པོ་ (Tsan dan dkar po)	Wood	W	42.11	12.49
Thymeliaceae *Aquilaria agallocha* Roxb.	Agarwood ཨ་ག་རུ་ (A ga ru)	Wood	W	42.11	6.85
Zingiberaceae *Amomum subulatum* Roxb.	Greater cardamom ཀ་ཀོ་ལ་ (Ka ko la)	Fruit	C	36.84	11.11
Zingiberaceae *Elletaria cardomomum* (L.) Maton.	Cardamom སུག་སྨེལ་ (Sug smel)	Fruit	C	42.11	8.20
Zingiberaceae *Hedychium spicatum* Ham.ex. Sm	Spiked ginger lily གཱ་སྐྱེ་ (Gah-kyah)	Root	C	31.58	14.30

* Both forms of *gur-gum*, *C. tinctorius* and *C. sativus*, were collected. See text for details.

4.2.3. Free radical scavenging activity

Following the methods of Cotelle *et al.* (1996), 3.0 mL 100μM 1,1 diphenyl-2-picryl-hydrazyl (DPPH) (Sigma Chem.Co.) in methanol and 0.5 mL of different plant extract concentrations in methanol stood at room temperature for 10 minutes, with absorbance read at 517 nm. Using the linear portion of the dose-response curve obtained with ascorbic acid, an IC_{50} (inhibitory concentration at 50%) was obtained as follows: base line absorbance was set with the DPPH solution alone, and 100% change in absorbance established when DPPH became completely bleached. The concentration of ascorbic acid which caused 50% change in absorbance was calculated using linear regression. Plant activity was presented as concentration (ppm) required to attain antioxidant activity identical to that of half of ascorbic acid (Ursis *et al.*, 1994). Because of possible interference due to plant pigmentation, plant concentrations in the absence of DPPH were measured and their absorbance as a function of percent volume was subtracted from previously obtained values. Quercetin, catechin and epicatechin (Sigma) were used as reference flavonoids.

4.2.4. Low-density lipoprotein preparation

Human LDL in solution containing 0.15 NaCl and 0.01% EDTA (Sigma) was diluted in 1 mM phosphate buffer solution (PBS) (0.175 g Na_2HPO_4 and 1.05g $Na_2HPO_4 \cdot H_2O$ made up to 1000 g with millipore water, pH adjusted to 7.4 with 1M NaOH and treated with Chelex® 100 Chelating Ion Exchange Resin (BioRad Lab., CA.) for one hour to remove any potential contaminating metals). LDL-PBS solution was passed through a Sephadex PD-10 column (Pharmacia Biotech, Uppsala) to remove most of the NaCl and EDTA, and lipoprotein concentration was estimated with the Sigma Diagnostics protein assay kit (Sigma) using bovine serum albumin (Sigma) as a standard.

4.2.5. Thiobarbituric acid reactive substances (TBARS) analysis

Determination of TBARS follows the procedure of Vaya *et al.* (1997) with the following modifications. For each sample, six test tubes containing LDL (0.10 mg

protein/LDL), plant extract (2.5 ppm prepared in 50% v/v MeOH), $CuCl_2$(5μM prepared in H_2O immediately prior to the assay) made up to 1 mL with PBS were incubated in a shaking water bath at 37°C and two tubes removed at 0, 90 and 180 minutes. Prior to the addition of $CuCl_2$, LDL was incubated for 1 hour with plant extracts. To stop oxidation, tubes were put on ice and 3 mM EDTA and 100μM BHT added. Once cooled, 1.0 mL TBARS solution (0.375 g thiobarbituric acid (Sigma) in 2.5 mL 2.0 N HCL, 15 mL trichloroacetic acid (Sigma) and millipore water filled to 100 mL) was added, the mixture vortexed and heated in boiling water for 20 minutes. The tubes were cooled to room temperature, centrifuged at 478 g for 10 minutes, and the absorbance of the supernatant recorded at 532 nm versus a water blank using a Beckman DU®-40 spectrophotometer. Concentration of TBARS was calculated using a standard curve of malondialdehyde (MDA), generated by the acid hydrolysis of 1,1,3,3-tetraethoxypropane (0.5-16 nmol/mL)(Sigma) and final results expressed as nmol MDA equivalents/0.1mg LDL protein. Trolox®, (8.95μM) a water soluble analogue of vitamin E, and ascorbic acid (12.71 μM), and a 50/50 (v/v) mixture (4.48 μM Trolox® and 6.36 μM ascorbic acid) were used as standards.

4.2.6. Conjugated dienes formation

Continuous monitoring of LDL by measuring the increase in absorbance at 234 nm follows the procedures of Esterbauer *et al.*, (1990) with the following modifications. In a semimicro quartz cuvette, LDL (0.01 mg protein/LDL), plant extract (1 ppm prepared in 50% v/v MeOH), $CuCl_2$(5μM prepared in H_2O immediately prior to the assay) made up to 1 mL with PBS was mixed and absorbance read every 10 minutes for the first 100 minutes, then every 20 minutes using a Beckman DU®-640 spectrophotometer until an increase in absorption (propagation phase) could be measured. Base line absorbance was set to 0.0 immediately after addition of $CuCl_2$ and lag time determined graphically by the intersection of the tangents of the propagation phase curve with the lag phase. Trolox®, (3.58μM) a water soluble analogue of vitamin E, and ascorbic acid (5.08 μM), and a mixture (1.79 μM Trolox® and 3.18 μM ascorbic acid) were used as standards.

4.2.7. Statistical analysis

Results of DPPH and TBARS assays are expressed as mean ± standard error from duplicate samples of three assays. Data from conjugated dienes are expressed as mean ± SEM from a single sample of three assays. A one-way ANOVA established at $P<0.05$ significance was performed for DPPH and conjugated dienes data; a one-way ANOVA with a repeated measure on one factor (time), established at $P<0.05$ significance was performed on TBARS data. Post-hoc multiple comparisons using Tukey analysis was performed to assess for differences in the means. Pearson correlation was used to compare data sets. Statistical analysis was performed using SAS software, version 6.11.

4.3. Results

4.3.1. Free Radical Scavenging Ability

The 1,1 diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging ability of crude methanol plant extracts is presented in descending order of antioxidant activity, represented as an increase in the 50% inhibitory concentration (IC_{50}) as calculated from data obtained from ascorbic acid (Figure 4.3.1a.). The data for the flavonoids quercetin, catechin and epicatechin are also included. Of the 14 crude plant extracts tested, only *T. chebula* had activity comparable to ascorbic acid and the standard flavonoids ($P<0.05$). *S. aromaticum*, *A. agallocha* and *S. album* also had notable antioxidant activity.

DPPH decolorizes from purple to yellow in the presence of an antioxidant. Two plant extracts, *B. arundinacea* and *C. sativus* failed to produce viable results because of photo-interference. Nodal silica from *B. arundinacea* was not a suitable substance for testing because of its mineral nature, and produced a brown hue. The principle pigment of *C. sativus*, α-crocin, a water-soluble carotenoid, interfered directly with the colorimetric analysis, producing a vivid orange. Since it was visually apparent that the DPPH had been completely quenched, the plant was selected for further testing in LDL oxidation experiments. Interestingly, *C. tinctorius*, a plant considered an inferior substitution for *C. sativus* in Tibetan medicine, had relatively low free radical scavenging activity.

An arbitrary cut-off point for plants to be partitioned was set at IC_{50}=150ppm (Figure 4.3.1a), which included *T. chebula* (6.59±0.11 ppm), *S. aromaticum* (30.16±0.79 ppm), *A. agallocha* (60.65±2.77 ppm), *S. album* (68.83±2.24 ppm), *A. subulatum* (135.33±2.18 ppm), *J. adhatoda* (140.60±0.91 ppm) and *M. fragrans* (146.82±0.44 ppm).

The DPPH free radical scavenging ability of hexane, chloroform, ethyl acetate and aqueous plant fractions is presented in alphabetical order, represented as an increase in the

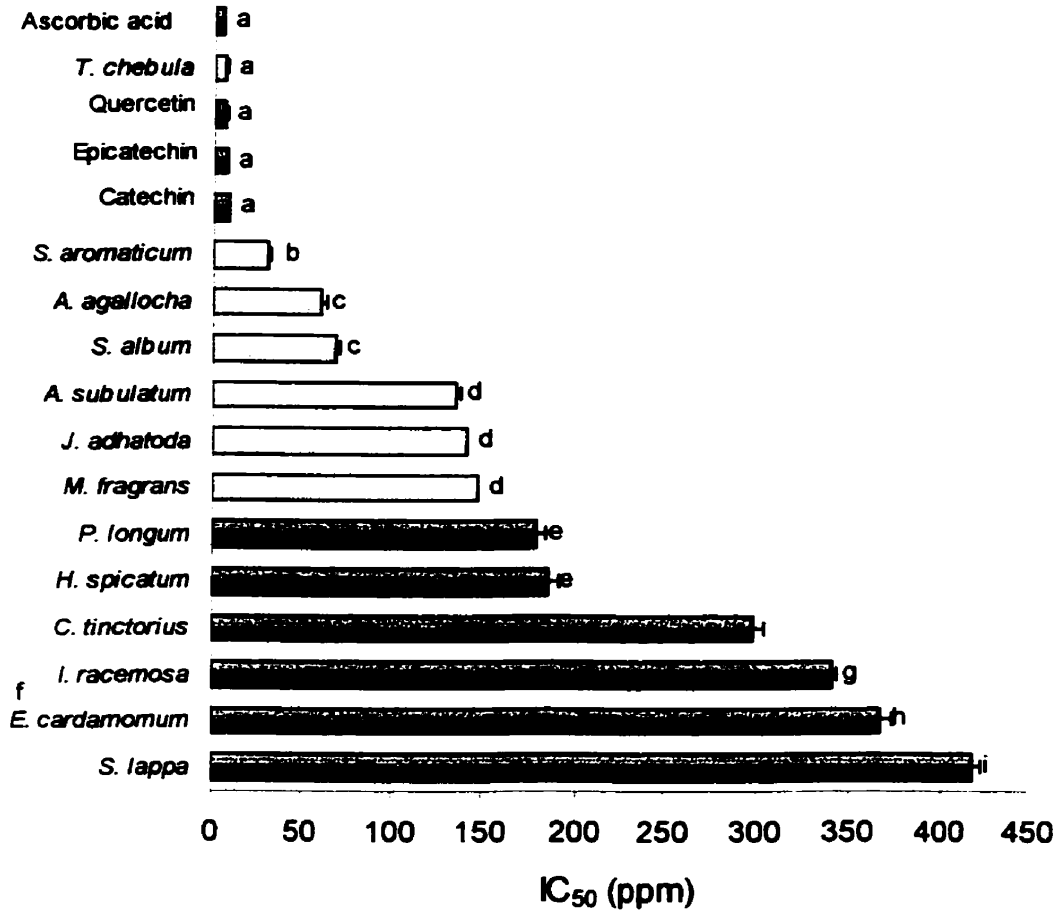

Figure 4.3.1a. Antioxidant activity of 13 Tibetan medicinal plants, as measured by the 1,1 diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging ability of methanol fractions. Bars represent mean ± SEM of duplicate samples of three assays; those with different letters are significantly different (P<0.05). Plant extracts are sorted in descending order of activity; those with relatively higher antioxidant activity, shown as light-coloured bars, were chosen for partitioning.

50% inhibitory concentration (IC_{50}) as calculated from data obtained from ascorbic acid (Figure 4.3.1b.). A post-hoc Tukey multiple comparisons analysis was performed within each plant group to determine the most active fraction. Labels from 'a' to 'd' therefore represent significant difference ($P<0.05$) among the four fractions of a single plant, with 'a' representing strongest activity.

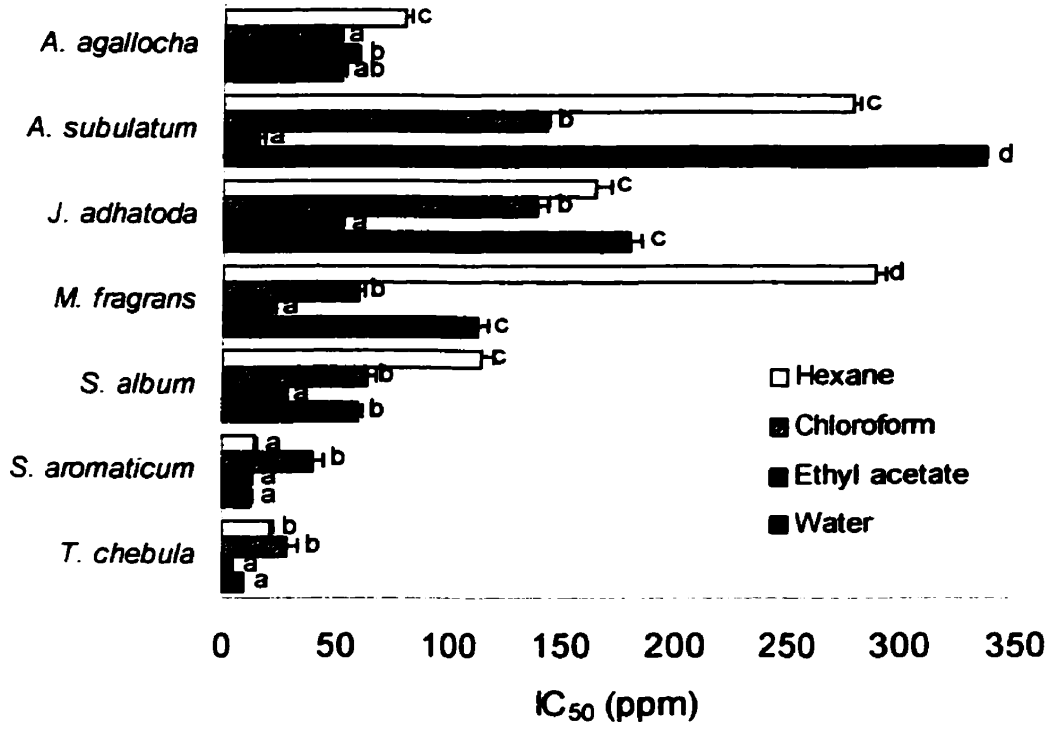

Figure 4.3.1b. 1,1 diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging ability of hexane, chloroform, ethyl acetate and aqueous fractions of plant methanol extracts. Bars represent means ± SEM of duplicate samples of three assays; those with different letters are significantly different ($p<0.05$) within plant groups. Fractions with the strongest antioxidant activity, represented by the letter 'a',were chosen for LDL oxidation experiments.

In four out of seven cases, the ethyl acetate fraction of plant extracts displayed strongest activity, a fraction in which glycosylated phenolics and flavonoids occur. Notable exceptions are the hexane fraction of *S. aromaticum* and the chloroform fraction of *A. agallocha*, which contain more lipophilic compounds. The plant fractions therefore selected for LDL oxidation experiments include the hexane fraction of *S. aromaticum*, the chloroform

fraction of *A. agallocha*, the ethyl acetate fraction of *A. subulatum*, *J. adhatoda*, *M. fragrans*, *S. album*, *S. aromaticum* and *T. chebula*, and the water fraction of *A. agallocha*, *S. aromaticum* and *T. chebula*.

4.3.2. Measurement of thiobarbituric acid reactive substances (TBARS)

The levels of TBARS were measured for plant fractions that had relatively high free radical scavenging activity at preincubation (0 minutes) and at two postincubation points (90 & 180 minutes), and are presented as stacked bars in decreasing order of activity (Figure 4.3.2.). Low levels of TBARS, expressed as nmol malondialdehyde equivalents / 0.1 mg LDL, indicate a low degree of lipid peroxidation and reflect the LDL protective effects of plant fractions. Trolox®, a water-soluble analogue of vitamin E, ascorbic acid and a combination of the two are also shown. Ascorbic acid displayed pro-oxidant activity, which has been shown previously in the presence of metal ions and minimally oxidized LDL (Otero *et al.*, 1997). A combination of ascorbic acid and Trolox® inhibited LDL oxidation comparable to Trolox® alone. Similar to ascorbic acid, the water fraction of *T. chebula* displayed pro-oxidant activity, with the majority of oxidation occurring within the first 90 minutes. Other seemingly pro-oxidant fractions include the water fraction of *S. aromaticum*, in which LDL oxidation occurred beyond 90 minutes, and the ethyl acetate fraction of *J. adhatoda*, in which oxidation increased steadily through time. These fractions did not differ significantly from the control or ascorbic acid.

Plant fractions that had significant LDL protective effects compared to the control and comparable to Trolox® included the hexane fraction of *S. aromaticum*, the chloroform fraction of *A. agallocha*, the ethyl acetate fractions of *A. subulatum*, *M. fragrans*, *S. album*, *S. aromaticum* and *T. chebula*, and the crude methanol extract of *C. sativus*. The water fraction of *A. agallocha* also inhibited LDL oxidation, but did not have significantly different activity compared to the control. An apparent pattern emerges upon examination of the results, such that fractions displaying relatively high LDL protective effects were the lipophilic hexane and chloroform fractions, followed by the ethyl acetate fractions. Water fractions on the other hand showed poorer, or even pro-oxidant activity.

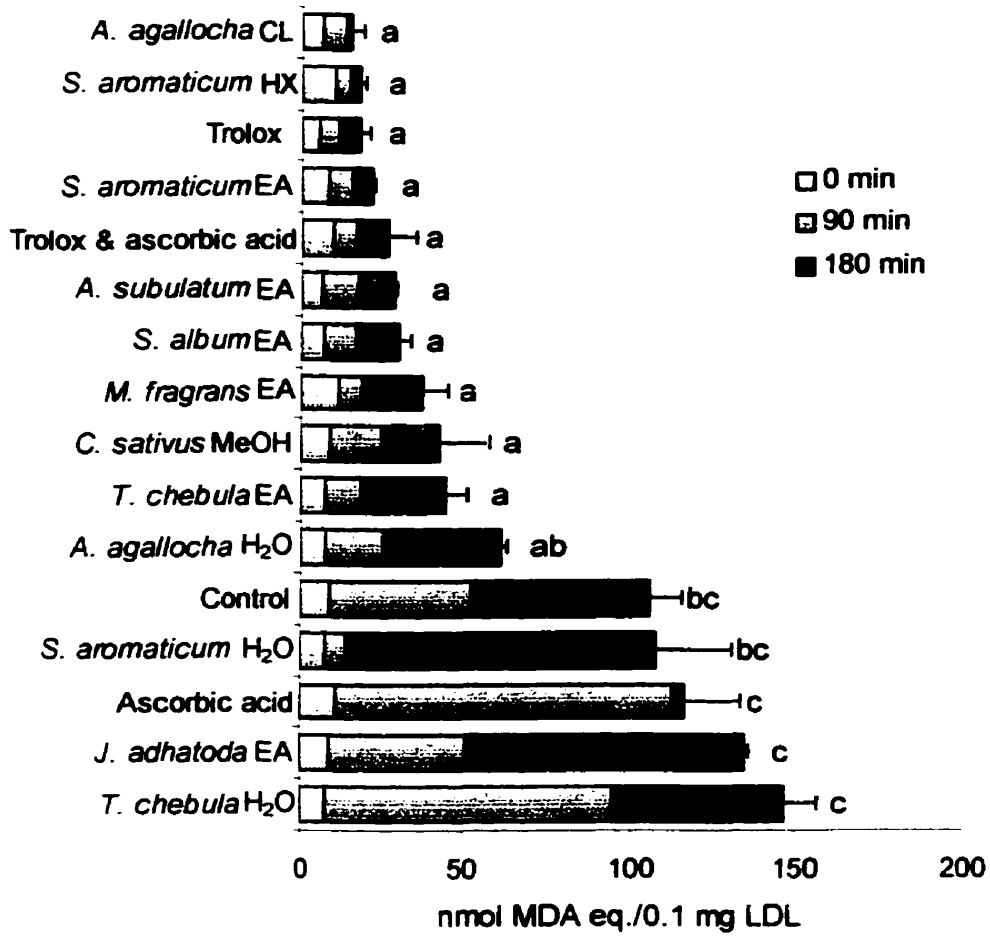

Figure 4.3.2. Low density lipoprotein (LDL)-protective effects of plant fractions measured as production of thiobarbituric acid reactive substances (TBARS) from Cu^{2+}-oxidized LDL incubated with 2.5ppm plant fractions, at preincubation (0 minutes), and postincubation (90 & 180 minutes). Bars represent means ± SEM of duplicate samples of three assays; those with different letters are significantly different ($P<0.05$). HX=hexane; CL=chloroform; EA=ethyl acetate; H_2O=water fractions; MeOH=methanol extract; MDA eq.=malondialdehyde equivalents.

4.3.3. Conjugated dienes formation

The effect of plant fractions having had high free radical scavenging activity on the lag time of conjugated dienes formation is presented in decreasing order of efficacy (Figure 4.3.3.). The hexane fraction of *S. aromaticum* had the longest lag time (1339.96±7.01 min), more than three times longer than Trolox® (431.02±21.19 min). The Trolox®-ascorbic acid

combination did not differ significantly from the control, whereas ascorbic acid alone again displayed pro-oxidant activity. The *T. chebula* water fraction also suggested pro-oxidant activity, although did not differ from the control significantly. Plants which showed an ability to prolong the protection of LDL from oxidation include the chloroform fraction of *A. agallocha* (255.29±18.84 min), the hexane fraction of *S. aromaticum*, the ethyl acetate fraction of *J. adhatoda* (252.41±5.77 min), *S. album* (230.28±9.48 min) and *T. chebula* (226.71±13.08 min), the water fraction of *A. agallocha* (223.92±12.80min), and the methanol crude extract of *C. sativus* (255.72±16.33 min).

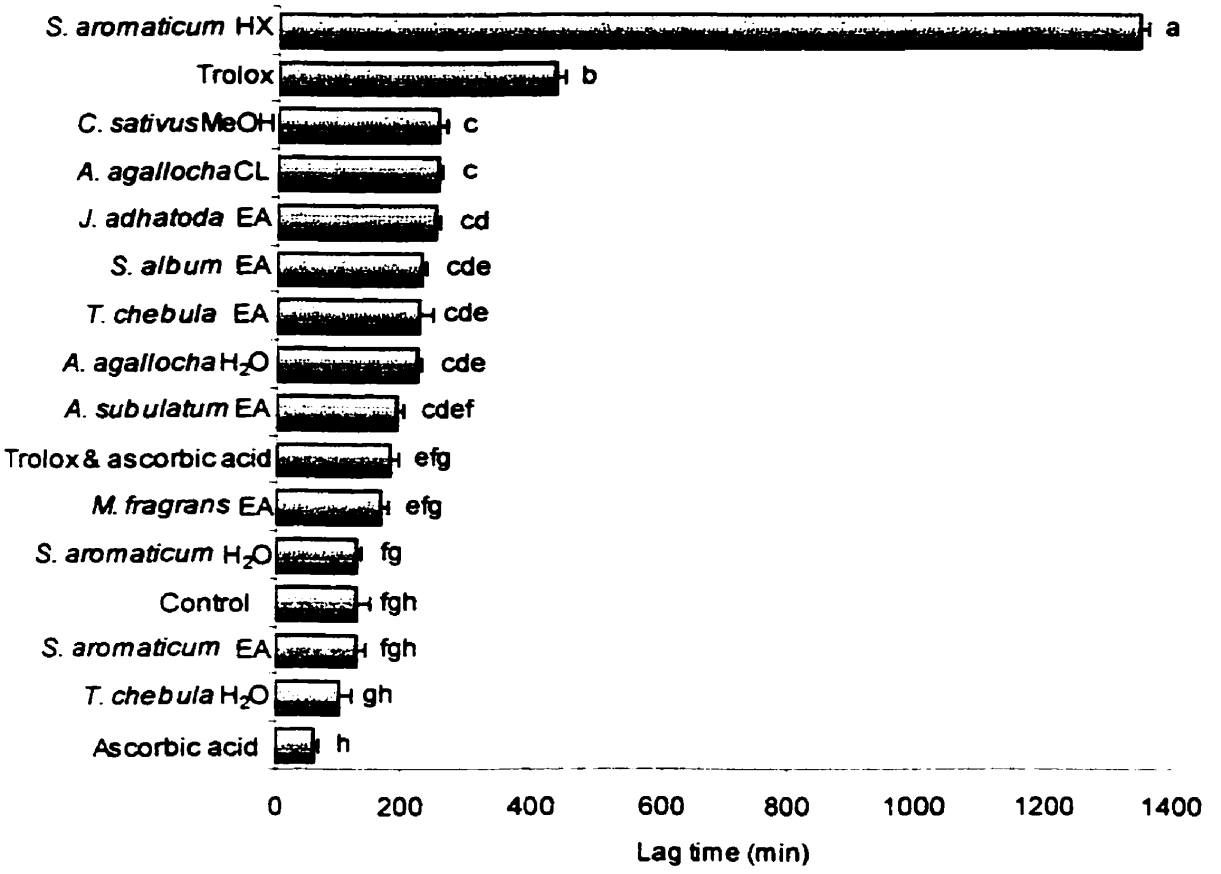

Figure 4.3.3. Low-density lipoprotein (LDL) protective effects of plant extracts (1ppm) measured as inhibition of conjugated dienes formation expressed as lag time (minutes). Oxidation was initiated with 5μM Cu^{2+} and absorbance continuously monitored at 234 nm at initially 10, then 20 minute intervals. Bars represent means ± SEM of three assays; those with different letters are significantly different (P<0.05). HX=hexane; CL=chloroform; EA=ethyl acetate; H_2O=water fractions; MeOH=methanol fraction.

4.3.4. Correlation among the three measures of antioxidant activity

Pearson correlation was performed to assess whether free radical scavenging activity of a compound would reflect its LDL-protective effects, and whether there is agreement between data obtained from the TBARS and conjugated dienes assays. Since these assays measure different parameters of a common reaction, a satisfactory comparison is expected (Slater, 1984).

TBARS production was measured at two time-points, whereas DPPH and conjugated dienes formation relate to the absolute antioxidant potential of a compound. As such, the only possible comparisons made were between lag time and DPPH; and absorbance of conjugated dienes formation at 90 and 180 minutes versus TBARS (Table 4.3.4.). Comparisons between DPPH results and conjugated dienes lag times were performed with the exclusion of *S. aromaticum* hexane fraction, considered an outlier (Inclusion would render r=-0.0787, P=0.82). The resulting coefficient, r=0.69, P=0.025, indicates a positive correlation, suggesting that free radical scavenging activity of a plant extract can predict its ability to protect LDL from oxidation.

Levels of TBARS were compared to the corresponding absorbance at 234 nm of conjugated dienes at 90 and 180 minutes. Ascorbic acid was excluded from the analysis since it had pro-oxidant activity in our assay as a result of its incubation with copper and minimally oxidized LDL (Otero *et al.*, 1997) and produces an outlier at 180 minutes (Inclusion would render 90 min; r=0.84, P=0.00003 and 180 min: r=0.61 P=0.011). Significant positive correlations were obtained for both 90 minutes (r=0.71, P<0.005) and 180 minutes (r=0.74, P<0.005) (Figure 4.3.4).

Table 4.3.4. Correlation coefficients and *P*-values between DPPH, TBARS and conjugated dienes data sets.

	Correlation coefficient and p-value		
	DPPH	**TBARS 90 min**	**TBARS 180 min**
Conjugated dienes	r=0.69, P=0.025[a]	r=0.71, P=0.0029[b]	r=0.74, P=0.0014[c]
TBARS 90 min			r=0.82, P=0.00011

[a] Compared with lag time of conjugated dienes without *S. aromaticum* hexane fraction, which was treated as an outlier.

[b] Compared with absorbance of conjugated dienes (234 nm) at 90 minutes. Ascorbic acid was excluded due to its pro-oxidant nature.

[c] Compared with absorbance of conjugated dienes (234 nm) at 180 minutes. Ascorbic acid was excluded due to its pro-oxidant nature.

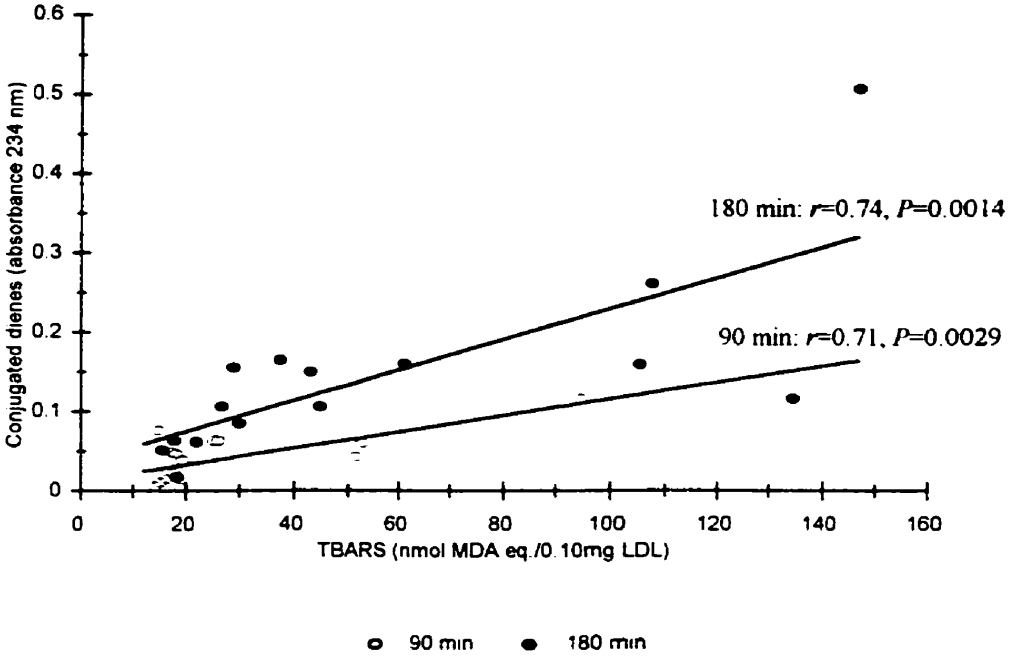

Figure 4.3.4. Correlation between TBARS levels (nmol MDA eq./ 0.10 mg LDL) and absorbance at 234 nm at 90 (○) and 180 (●) minutes of plant fractions (2.5 ppm for TBARS; 1.0 ppm for conjugated dienes) incubated with copper-oxidized LDL. Lines are drawn from least-squares regression.

4.4. DISCUSSION

Results showing the free radical scavenging ability and inhibitory effect of Tibetan medicinal plants on lipid peroxidation suggest their likelihood in contributing to the therapeutic effects of traditional drugs used for CVD. When LDL was submitted to copper-mediated oxidation, the addition of certain plant fractions in the media prolonged the consumption of endogenous α-tocopherol, increased the lag phase for the formation of conjugated dienes and decreased TBARS production. In particular, hydrophobic fractions such as hexane and chloroform, showed greatest antioxidant activity in protecting LDL from oxidation, perhaps because the active phytochemical constituents were readily incorporated into the LDL membrane. With the exception of *S. aromaticum* (Rajakumar & Rao, 1993), and *T. chebula* (Vani *et al.*, 1997) the plants included in the present study have not, to our knowledge, been subjected to the present assays.

4.4.1. Free radical scavenging ability

The methanol extract of *T. chebula* had the strongest free radical scavenging ability of the plants tested (Figure 4.3.1a). This confirms previous reports on the plant using superoxide, peroxide (Vani *et al.*, 1997) and hydroxyl (Maulik *et al.*, 1996) radicals. The plant's activity is attributed to its high tannin content (30-32%), which is of the pyrogallol type and yields chebulic acid (coumarin) and d-galloyl glucose (tannin) upon hydrolysis (Kokate *et al.*, 1995). Hydrolysable tannins of *T. chebula* may account for activity in both the ethyl acetate and aqueous fractions. Most gallotannins are soluble in ethyl acetate, whereas higher molecular weight gallotannins remain in the aqueous solution. Moreover, the fruit of *T. chebula* is rich in ascorbic acid (Barthakur & Arnold, 1991), a powerful hydrophilic free radical scavenger which may have contributed to the observed activity of the extract. Tannins, which posses properties characteristic of their constituent phenols, are well known for their ability to react with proteins (Okuda *et al.*, 1991). As a result of their affinity for proteins, tannin-containing drugs have limited application. Nevertheless, many plants used for medicinal purposes contain astringent tannins that are regarded as their active principles. These drugs are typically recommended as diuretics, antidiarrhoeals and

haemostatics, but have also been used for a wide range of other treatments, including arteriosclerosis and hypertension (Zhu *et al.*, 1997). Their therapeutic effect in these cases may be as inhibitors of reactive oxygen species and lipid peroxidation. Additional antioxidant phenols identified in *T. chebula* include vanillic acid, ferulic acid, caffeic acid and *p*-coumaric acid (Kim *et al.*, 1993) which could have contributed to the activity of the ethyl acetate fraction.

The three extracts with the next strongest free radical scavenging ability are known for their aromatic essential oils and are often incorporated into incenses and medicines in the Orient. The hexane fraction of *S. aromaticum* would likely contain eugenol and isoeugenol, in addition to several dozen aliphatic aromatic and heterocyclic terpenoid compounds (Bruneton, 1995). Those of *A. agallocha* likely contain the sesquiterpenes agarospirol, agarol, and half a dozen selinanic furanoids (Nakanishi *et al.*, 1981). Eugenol and isoeugenol, major phenylpropanoid components of *S. aromaticum* essential oil, have confirmed free radical scavenging activity against the hydroxyl and superoxide radical. Isoeugenol, due to the presence of a conjugated double bond, was found to be more potent than eugenol as a scavenger and in inhibiting lipid peroxidation (Rajakumar & Rao, 1993). Eugenol also appears to act as an *in vivo* antioxidant as demonstrated in rats intoxicated with CCl_4 (Kumaravelu *et al.*, 1995). No known antioxidant activity has been reported for *A. agallocha*, although neuroactive properties have been investigated (Okugawa *et al.*, 1996, 1993).

The antioxidant activity of tannins and phenolics such as flavonoids is essentially due to the phenol group, which can react with a free radical to form the phenoxyl radical. The presence of certain other functional groups and double bonds ultimately affects a phenol's stability as an antioxidant (Cuvelier *et al.*, 1992). Tannins and flavonoids such as kaempferol, myricetin and quercetin in *S. aromaticum* would likely account for antioxidant activity of the ethyl acetate and water fractions. Tannins in angiosperms are associated with woody tissues, and since the wood of *A. agallocha* and *S. album* is used medicinally, it is likely that free radical scavenging ability of these extracts was due to tannins.

Flavonoids such as alpinetin, cardamomin and subulin in *A. subulatum* (EA), and

cyanidin and delphinidin in *M. fragrans* (EA) may confer antioxidant activity. As with *S. aromaticum*, *M.fragrans* contains eugenol and isoeugenol, albeit in lower amounts. The *M. fragrans* hexane fraction however showed relatively poor antioxidant activity. The high lipid content (e.g. myristic acid) of the fraction caused turbidity in solution and interfered directly with absorbance readings. The solution, when slightly heated, became clearer, although it could have regressed to greater turbidity during DPPH incubation at room temperature.

4.4.2. Measurement of TBARS

It is well established that a great variety of aldehydes are formed through the decomposition of lipid hydroperoxides. Approximately 90% of TBARS is free malondialdehyde, which produces a red species absorbing at 535 nm (Esterbauer *et al.*, 1987). Reactive malondialdehyde interacts with the functional groups of apolipoprotein B (apo B), and contributes to the altered functional properties of oxidized LDL, ultimately leading to cholesterol loading of macrophages (Haberland *et al.*, 1982; Esterbauer *et al.*, 1990).

Measurement of TBARS produced at preincubation (0 min) and at 90 and 180 minutes postincubation showed that the hydrophobic fractions of *S. aromaticum* and *A. agallocha*, the ethyl acetate fractions of *S. aromaticum*, *A. subulatum*, *S. album*, *M. fragrans* and *T. chebula*, and *C. sativus* extract did not produce significantly more TBARS than Trolox® during LDL oxidation (Figure 4.3.2.). Conversely, *J. adhatoda* (EA) and the aqueous fractions of *S. aromaticum* and *T. chebula* displayed pro-oxidant activity, and were comparable to the control or ascorbic acid, a known pro-oxidant in the presence of metal ions and minimally oxidized LDL (Otero *et al*, 1997). Our results indicate that LDL in the presence of ascorbic acid was completely oxidized within 90 minutes. Pro-oxidant activity has been demonstrated in some polyphenols in which high concentrations (25-100μM) accelerated hydroxyl radical production *in vitro* (Canada *et al.*, 1990). Depending on experimental conditions, Laughton *et al.* (1989) found that the phenolics quercetin and myricetin, two compounds likely occurring in the aqueous fraction of *S. aromaticum*, can have pro-oxidant activity. The pro-oxidant nature of certain phenols has been theorized as

follows. When phenols are used at low concentrations, a low concentration of secondary phenoxyl radicals are produced from redox reactions. These phenoxyl radicals are likely to react with the primary radical and quench it, resulting in a stoichiometry of two radicals per phenol. High phenol concentrations therefore result in equally high levels of secondary phenoxyl radicals, and the ensueing stoichiometry is reduced to one, as a phenoxyl radical is equally likely to react with another phenoxyl radical as with the primary radical (Robinson *et al.*, 1997). Pro-oxidants in *T. chebula* may be attributed to certain polar phenolics or tannins, but may equally be due to the high levels of ascorbic acid found in the fruit (Barthakur & Arnold, 1991).

The apparent pro-oxidant nature of *J. adhatoda* as measured by level of TBARS is inconsistent with data obtained from conjugated dienes formation (Figure 4.3.3.). Biochemical investigations of *J. adhatoda* have focused on the abortifacient alkaloid vasicine and a dozen antiviral arylnaphthalide lignans (Asano *et al.*, 1996). Little information on other constituents that may affect LDL peroxidation exists. Our results suggest the presence of a compound in *J. adhatoda* that reacts with Cu^{2+} and/or LDL to release a TBA reactive substance separate from its ability to increase LDL resistance to peroxidation as measured by lag time.

4.4.3. Conjugated dienes formation

The primary products of lipid peroxidation are conjugated lipid hydroperoxides which can be directly measured in the UV spectrum of the aqueous LDL solution (Esterbauer *et al.*, 1989). Our results show that the hexane fraction of *S. aromaticum* had a lag phase more than three times longer than that of Trolox® (Figure 4.3.3.). This activity may be attributed to the presence of eugenol, which has been previously shown to inhibit lipid peroxidation at the level of initiation and propagation phase (Nagababu & Lakshmaiah, 1992). This property is evident as shown in Figure 4.4. where the rate (slope) of peroxidation during the propagation phase is lower compared to the control and Trolox®. Halliwell & Gutteridge (1989) emphasized the importance of site-specific generation of

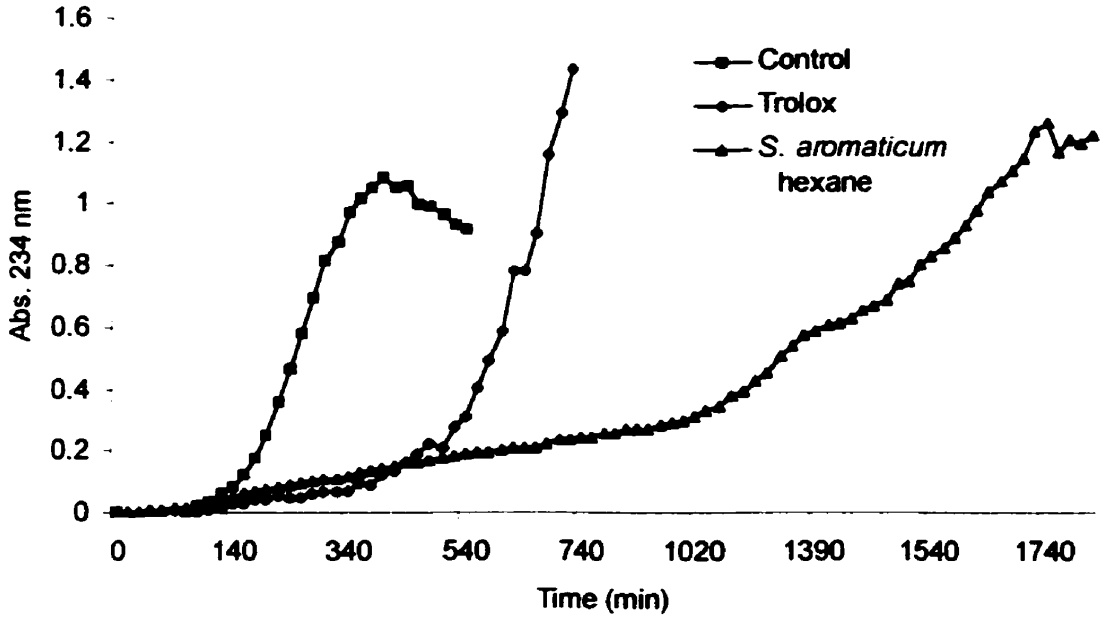

Figure 4.4. Continuous monitoring of absorbance at 234 nm for conjugated dienes formation of LDL exposed to copper-catalysed oxidation (control) in the presence of Trolox® and the hexane fraction of *S. aromaticum*. Data shown are representative of a single experiment and represent selected data obtained from Figure 4.3.3. Repeated measurements produced similar results.

oxygen radicals at metal binding sites in membranes. In view of its lipophilic nature, eugenol is able to reach these sites and interfere with peroxidation by propagation radicals generated at these sites. Lipophilic antioxidants such as eugenol and isoeugenol, therefore offer greater protection against peroxidation because of their ability to interact with lipids. In a related *in vivo* study, Deans *et al.* (1995), found that *S. aromaticum* essential oils protected polyunsaturated fatty acids in the liver and retina of elderly mice from oxidation.

All plant fractions, with the exception of the ethyl acetate fractions of *A. subulatum*, *M. fragrans*, *S. aromaticum* and the water fractions of *S. aromaticum* and *T. chebula*, had lag times significantly longer than the control ($P<0.05$) (Figure 4.3.3.). The methanol extract of *C. sativus* significantly decreased LDL susceptibility to oxidation in our study. Antioxidant activity of *C. sativus* has been previously demonstrated in mice (Nair *et al.*, 1992) and humans (Verma & Bordia, 1998), likely due to its high carotenoid content, particularly crocetin and crocin. It is thought that the consumption of *C. sativus* in Spain, which also inhibits platelet aggregation (Nishio *et al.*, 1987), is related to the low incidence

of cardiovascular disease despite a diet rich in fats and calories (Grisolia, 1974).

Tannins, which are able to inhibit lipid peroxidation *in vitro* (Okuda *et al.*, 1991) may account for the LDL protective effects demonstrated by *A. agallocha* (H_2O), *S. album* (EA) and *T. chebula* (EA), the major tanniniferous plant of India. Tannins are also able to bind directly to LDL *in vitro* (Vinson *et al.*, 1995), which has been demonstrated for phenolic substances in red wine (Fuhrman *et al.*, 1995).

4.4.4. Correlation among measures of antioxidant activity

According to Zhang *et al.*, (1994), the two most important parameters for measuring lipid peroxidation are the formation of TBARS after 180 minutes and lag time. The former relates to the maximal capacity of lipid peroxide formation of a fraction, while the latter displays its antioxidant potential. Our results show consistency between TBARS levels and corresponding absorbance at 234 nm, suggesting that most plants, with the possible exception of *J. adhatoda*, displayed no irregular mechanisms in their ability to inhibit peroxidation. DPPH data also correlated positively with lag time, with *S. aromaticum* (HX) excluded as an outlier, suggesting that general free radical scavenging activity can successfully predict an extract's ability to inhibit peroxidation.

4.4.5. Possible mechanisms of action

In contrast to isolated compounds (e.g., ascorbic acid), any activity attributed to a crude extract stems from a multicomponent reaction. Typically, bioactivity of mixtures is greater than purified components, indicating synergistic or additive effects (e.g., Packer *et al.*, 1999). A common problem in dealing with multicomponent preparations of natural origins is not only attributing activity to a principle compound, but also determining whether co-occurring compounds facilitate or enhance its intestinal absorption and/or metabolic fate. Antioxidant activity of individual plant extracts in our study may be attributed to a single active principle, but is more likely orchestrated by several interacting compounds which might ultimately affect *in vivo* activity and reduce possible side effects.

Different possible mechanisms by which plant extracts exert their LDL protective

effects include metal-chelating potential of polyphenols, which are effective in preventing metal-catalysed formation of initiating radical species, and in protecting LDL from iron- and copper-mediated free radical production (Laughton *et al.*, 1991). Moreover, polyphenols may scavenge lipid alkoxyl and peroxyl radicals and/or regenerate α-tocopherol through the reduction of the α-tocopheroxyl radical (reviewed in Rice-Evans, 1995).

Phytochemicals can also exert a cardioprotective effect other than as antioxidants. For example, *M. fragrans* possesses hypocholesterolemic activity (Ram *et al.*, 1996) and reportedly prevents the accumulation of cholesterol, phospholipids and triglycerides in the livers and hearts of hypercholesterolemic rabbits fed *M. fragrans* extract (Sharma *et al.*, 1995). Additional antithrombotic properties of *M. fragrans* include inhibition of platelet aggregation (Janssens *et al.*, 1990) through its influence on prostaglandin biosynthesis (Rasheed *et al.*, 1984). The bioactive compounds in these cases were found to be eugenol and isoeugenol. Cholesterol synthesis is reduced in humans fed safflower (*C. tinctorius*) oil, a rich source of linoleic acid (18:2 n-6) (Cox *et al.*, 1998). Moreover, antioxidant serotonin derivatives have been isolated from safflower oil cake (Zhang *et al.*, 1997). Coronary vasodilating activity has been reported in *Saussurea lappa* (Shoji *et al.*, 1986a) and *Piper longum* (Shoji *et al.*, 1986b), two plants which had relatively poor antioxidant activity in the present study. *H. spicatum* reduces blood pressure in dogs (Shaw, 1980), *E. cardamomum* is hypotensive in rats (Haranath *et al.*, 1987), *P. longum* oil is hypocholesterolemic in experimentally induced hypercholesterolemic mice (Bao & Wu, 1992), as is *T. chebula* in rats (Khanna *et al.*, 1993). The low antioxidant activity in tested plants therefore does not necessarily invalidate their use as cardioactive medicines.

4.4.6. Multicontextual uses of plants

The majority of the plants included in this study are common spices. Because several indigenous cultures gauge a plant's medicinal efficiency on taste, olfaction, appearance and texture (Johns, 1990, 1994; Etkin, 1996), it is no surprise that spices contribute an important component to the development of local pharmacopoeias. As such, biomedical investigations have shown several spices to be rich sources of antioxidants and other bioactive principles,

and support their therapeutic use to promote health (Nakatani, 1997).

Spices in medicines has been viewed as mechanical and/or symbolic adjuvants, vehicles or facilitators of other ingredients, and since these plants are used to treat a wide range of disorders, any activity attributed to the composite medicine is usually presumed to be due to other ingredients (Etkin, 1986). Indeed, plants selected for analysis in the present study occur in other Tibetan medicines prescribed for a range of different disorders. However, Tibetan and Ayurvedic medicinal concepts have long understood spices as important therapeutic agents (Handa, 1998) and they have either been used to support primary ingredients or as primary constituents themselves.

4.4.7. Bioscientific implications of Tibetan medicine

Tibetan medicine is a holistic therapeutic system based on the fundamental concept of the three humours: wind (*rlun*), bile (*mkris-pa*) and phlegm (*ban kan*). When the humours are in equilibrium, health is maintained. Disease occurs in the event of an imbalance, caused by factors such as seasonal changes, improper diet, or wrongful behaviour. Each humour is reflective of the three body-mind constitutions, each associated with particular health needs and behaviors, dietary and lifestyle factors, and predisposition to certain diseases and symptoms. Each has its own method of treatment in terms of nutrition, behavior, medicine and external treatments (Finckh, 1984; 1982). Because of the complex concepts, diagnosis and treatments of disease, and because there are seven recognized types of cardiovascular disease (Dorjee & Richards, 1985), persons suffering identical disorders may be given different medicines and treatments. The nature of Tibetan medicine clearly entails certain difficulties when conducting biomedical investigations. Nonetheless, the efficacy of Tibetan medicine has been demonstrated for disorders such as intermittent claudation (Schräder *et al.*, 1985; Smulski, 1991; Drabaek *et al.*, 1993), atherosclerosis (Wójcicki *et al.*, 1988; Gieldanowski *et al.*, 1992) and arthritis (Ryan, 1997). Therapeutic activity in these medicines, which usually are mixtures of several different plant ingredients, has been attributed in part to the high levels of heparinoids and flavonoids contained therein, described as a "powerful mixed-plant antioxidant source" (Fishman, 1994b).

Compound medicines are a fundamental concept of Tibetan medicine, and present certain obstacles for biomedical investigations. The difficulties in ascribing the biological effects to one of many constituents of a single plant, are further confounded when considering constituents of several other plants. These notwithstanding, the merits of compound medicines should be further investigated in light of potential synergistic, additive and/or antagonistic effects which may result as different constituents interact. For instance, bioscientific investigations concluded that *P. longum* and *P. nigrum* in compound medicines significantly enhanced the bioavailability of bioactive principles in other medicines (Handa, 1998; Atal *et al.*, 1981). Similarly, as antioxidant activity from an extract may be greater than from an isolated compound, the activity of a plant mixture would conceivably be greater than the individual plant. Hence, although antioxidant activity of Tibetan medicinal plants has been verified in this study, their activity may be enhanced, whether by increased antioxidative potential, increased bioavailability or reduce toxicity, in biological systems.

4.4.8. Conclusion

Because oxidatively modified LDL is implicated in the pathogenesis of atherosclerosis, treatment with antioxidants could conceivably retard the progress or actually regress the atherosclerotic lesion. It has been shown that oxidation of polyunsaturated fatty acids (PUFA) in LDL is preceded by the sequential loss of its endogenous antioxidants such as α-tocopherol and carotenoids (Esterbauer *et al.*, 1991). Supplementation with antioxidants, particularly those that demonstrate lipoprotein-binding, can regenerate or conserve endogenous antioxidants by scavenging radicals in aqueous compartments. Although the issue of efficacy and complexities of compound medicines have been raised, our results show that medicines which contain plants with high antioxidant activity, particularly *A. agallocha*, *A. subulatum*, *J. adhatoda*, *M. fragrans*, *S. album*, *S. aromaticum* and *T. chebula*, may aid against the development of CVD. Bioavailability and access to target tissues are considerations needed to assess the efficacy of a drug. Although there is limited available information on bioavailability of phenolics, it has been shown that flavonoids are present in plasma in high enough concentrations to have a biological effect

in vivo (Bourne & Rice-Evans, 1999; Hollman, 1997; Manach *et al*, 1995).

Our results show that several (53.85%) plants most often occurring in Tibetan medicines used for CVD have strong or moderate antioxidant activity. Ingestion of these botanicals, particularly *S. aromaticum*, may provide increased resistance to oxidative stress. If we assume that Tibetan highlanders have access to traditional medical treatments in the event of disease symptom onset, and that medicines are consumed as part of a dietary regime, then CVD onset may decidedly be curbed. Similarly, if plants rich in antioxidants are spices ingested on a regular basis, they may possibly act as cardioprotectants and contribute to the low CVD incidence among Tibetan highlanders.

5. GENERAL CONCLUSIONS

The present study has investigated the antioxidant activity of 14 plants commonly incorporated into Tibetan medicines used for cardiovascular disease, and was undertaken to explore possible dietary/medicinal factors that may contribute to the reportedly low incidence of cardiovascular disease among Tibetan highlanders despite high haematocrit levels. Although the factors affecting Tibetan susceptibility to CVD are complex and multi-faceted, we have concentrated on dietary and environmental considerations. A review of possible sources of oxidative stress included polycythemia, increased haemoglobin, hypoxia and increased exposure to ionizing radiation. Nutritional risk factors included a diet high in mammalian saturated fats and sodium and low in fruits and vegetables. Possible cardioprotective dietary elements include hypocholesterolemic activity of barley, their staple food, and flavonoid antioxidants in tea, their staple beverage. This was the first known study to address this issue in terms of dietary behaviour and ingested botanicals.

This study demonstrated the antioxidant activity of several plant extracts and fractions in scavenging the stable free radical 1,1 diphenyl-2-picryl-hydrazyl (DPPH) and increasing resistance to LDL exposed to Cu^{2+}-catalysed oxidation. Free radical scavenging activity was strongest in crude methanol extracts of *T. chebula* > *S. aromaticum* > *A. agallocha* = *S. album* > *A. subulatum* = *J. adhatoda* = *M. fragrans*. These extracts were partitioned into hexane, chloroform, ethyl acetate and aqueous fractions and retested in the DPPH assay to determine the most active fractions. These were subsequently analysed for ability to inhibit human LDL oxidation. Measurement of TBARS production and lag time for conjugated dienes formation showed that the *S. aromaticum* hexane fraction was most effective in increasing resistance of LDL to oxidation. Lag time was more than three times longer than that of Trolox®, a water-soluble analogue of vitamin E. Activity was likely attributed to eugenol and isoeugenol, the dominant constituents of *S. aromaticum* essential oil. In view of its lipophilic nature, these phenylpropanoids are likely to integrate into the LDL membrane and interfere with peroxidation by propagation radicals generated at membrane metal-binding sites. Other effective fractions were the *A. agallocha* chloroform

fraction and water fractions, *S. album* and *T. chebula* ethyl acetate fractions and *C. sativus* crude extract. Activity may be attributed to carotenoids in *C. sativus*, and to tannins and phenolics in *A. agallocha*, *S. album* and *T. chebula*.

All experiments correlated positively with each other, suggesting consistencies in antioxidant activity and free radical scavenging ability among plant extracts. DPPH radical scavenging ability correlated with lag times (r=0.69, P=0.025), as did TBARS levels with absorbance from conjugated dienes at corresponding time points (90 min.: r=0.71, P<0.005; 180 min.: r=0.74, P<0.005).

There are limitations to the present research design. Antioxidant activity shown in this study is limited to the quenching of radicals generated by the experimental conditions, and may not necessarily apply to other radicals or reactive oxygen species. Furthermore, the concentrations used are not indicative of those that would occur *in vivo*. Normolipidemic subjects have a plasma LDL concentration of about 2.5 mg/mL, and Hollman (1997) has estimated quercetin levels to reach up to 1μM (≈0.338 ppm) in human plasma. The concentration of extracts used in this study (1.0 ppm extract/0.10 mg LDL/mL) are higher than what would normally occur in biological systems. Moreover, data on the absorption and metabolism of phenolics and terpenes, both at the dietary and pharmacological levels, is scarce, and any antioxidant activity shown *in vitro* may not necessarily be effective *in vivo*. Nevertheless, several studies have found that flavonoids are absorbed at high enough concentrations to have a biological effect (Bourne & Rice-Evans, 1999; Hollman, 1997; Manach *et al.*, 1995).

Further limitations of this study concern the context in which the medicines are used. Tibetan medicine is usually prepared in the form of pills (powders, decoctions, medicated oils and butters are also noted) that contain a number of plant ingredients. Pills are slightly masticated before being swallowed with hot water. We have analysed only a fraction of plant ingredients that occur in any given medicinal pill prescribed for the treatment of CVD. Any synergistic, antagonistic or additive effect of these multi-component mixtures were therefore not considered. Moreover, by reviewing Tibetan medical lexicons for disorders related to CVD, we have approached Tibetan medicine as a cataloging system of discrete

diseases and attendant treatments rather than one of identifying disorders on the basis of individually specific social, emotional and environmental characteristics, a practice that is the hallmark of humoral theory (Janes, 1995). Our research design however, necessitated a satisfactory means of interpreting Tibetan medical theory in the context of contemporary biomedicine.

It should also be noted that efficacy of indigenous medicines using biomedical standards need not be necessarily validated since the concept of efficacy is culturally constructed. The efficacy of a drug may be viewed as physical, semiotic, or social, depending on the point of view of the user or investigator. Although biomedicine tends to see the function of plant medicines as being able to produce a desired physical response, the plant's user may see efficacy in decidedly nonphysical terms. In such cases, the usual interpretive tact is to assert that the bioactive component of the plant is a latent effect that somehow explains the plant's persistence in the pharmacopoeia. Such non-western medical systems are clearly based on concepts of disease and health differently than those perceived by western biomedicine (Etkin, 1986, 1988b, 1996).

Future research directions may be targeted towards further biomedical validation of Tibetan medicines, and towards the elucidation of low CVD incidence among Tibetan highlanders. Concerning the former, it is suggested that plants used to treat CVD be subjected to additional *in vitro* assays which relate to other cardiovascular mechanisms (e.g. hypocholesterolemic, hypotensive and vasodilating activity). In addition, *in vivo* studies using animal models are needed to investigate the pharmacokinetics characteristics of active constituents. Ultimately, compound medicines should be investigated to observe possible phytochemical interactions. Although the difficulties entailed are numerous, analysis of compound medicines and their biochemical fate may lead to a better understanding of holistic medicines in general.

The effect of ingested botanicals in reducing the risk of CVD among Tibetan highlanders can be accomplished by quantifying dietary intake of plants in a population sample. Dietary influences can be observed by measuring blood lipid and cholesterol levels, antioxidant status and related parameters through human dietary intervention trials.

Biochemical analysis of dietary and medicinal plants which are ingested relatively frequently can then be related to their biological effect to establish a clearer cause and effect association.

In applying biomedical standards to Tibetan medicine, we have in part validated its therapeutic efficacy for the treatment of CVD. Furthermore, if we assume Tibetan highlanders have access to traditional medical treatments in the event of disease symptom onset, and that medicines are consumed as part of a dietary regime, then CVD development may be controlled or negated. Similarly, if plants found to be rich in antioxidants are spices ingested on a regular basis, they may possibly act as cardioprotectants and contribute to the low CVD incidence reported by researchers. Biomedically speaking however, interpretations as to efficacy and validity are subjective when viewed in a cultural context. Poor antioxidant activity of a plant does not necessarily invalidate it's use since efficacy is a culturally constructed concept, and/or the plant may possess other cardioprotective elements which affect other biomechanisms. Moreover, it is possible that some plants are added to medicines to modify the activity or bioavailability of other components, and may not, in themselves, be cardioactive.

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6. APPENDICES

Appendix I. Tibetan medicines used for the treatment of cardiovascular-related diseases. [1]Dash (1994); [2]Rinpoche (1976); [3]Tsarong (1986).

1) Agar-20[3] 20 ingredients

Composition:

Aquilaria agallocha
Syzygium aromaticum
Chaenomeles lagenaria
Myristica fragrans
Carthamus tinctorius
Melia composita
Inula racemosa
Bambusa arundinacea
deer antlers
Shorea robusta
Saussurea lappa
Strychnos noxvomica
Terminalia chebula
Fragaria nilgeernsis
Mesua ferrea
Emblica officinalis
Pterocarpus santalinus
rabbit's heart
elephant's gallstone
pearls or oyster shells.

Use and action: stabilizes *rLung* and blood
Neurological disorders
Hypertension
Fever of inflammation increased by *rLung*

Dosage: 2-3 grams daily at morning or night with hot or warm water.

2) Agar bco lna[1] 15 ingredients

Composition:

50g *Aquilaria agallocha* (stem)
20 g *Choerospondia axillaris* (fruit)
20 g *Santalum album* (stem)
30 g *Pterocarpus santalinus* (stem)
10 g *Myristica fragrans* (fruit)
20 g *Bambusa arundinacea* (exudate)
20 g *Crocus sativus* (flower)
20 g *Pegaephyton scapiflorum* (root)
30 g *Terminalia chebula* (fruit)
30 g *Terminalia belerica* (fruit)
30 g *Emblica officinalis* (fruit)
30 g *Inula racemosa* (root)
30 g *Sophora flavescens* (plant)
60 g *Amomum subulatum* (fruit)
10 g *Hedychium spicatum* (root).

Use and action: Chest and back pain
Diseases caused by *khrag rlung*.

Dosage: 500 mg in the evening with hot or warm water.

3) Agar brgyad pa[1,2] 8 ingredients

Composition:

50 g *Aquilaria agallocha* (stem)[1,2]
25 g *Myristica fragrans* (fruit)[1,2]
25 g *Choerospondia axillaris* (fruit)[1]; *Sterculia alata*[2]
25 g *Bambusa arundinacea* (exudate)[1,2]
10 g *Shorea robusta* (exudate)[1]; *Boswellia serrata*[2]
20 g *Saussurea lappa* (root)[1]; *Inula helenium*[2]
20 g *Terminalia chebula* (fruit)[1,2]
25 g *Messua ferrea* (flower)[1]; *Quisqualis indica*[2]

Use and action: Heart disease[1,2]
Pain in breast and liver[1,2]
Insanity [1,2]

Dosage: 500 mg in the evening with hot or warm water.

4) Agar so lna[1,2,3] 35 ingredients

Composition:

20 g *Aquilaria agallocha* (stem)[1,2,3]
20 g *Aquilaria sinensis* (stem)[1,2]; *Solms-Laubachia* sp.[3]
12.5 g *Cinnamomum parthenoxylon* (stem)[1]; *C. camphora*[2]; *C. cecidodaphne* [3]
17.5 *Santalum album* (stem)[1,2,3]
25 g *Primula secundiflora* (stem)[1]; *Pterocarpus santalinus*[2,3]
5 g *Myristica fragrans* (fruit)[1,2,3]
5 g *Syzygium aromaticum* (flower)[1,2,3]
35 g *Bambusa arundinacea* (exudate)[1,2,3]
15 g *Crocus sativus* (flower)[1,2]; *Carthamus tinctorius* [3]
15 g *Elettaria cardamomum* (fruit)[1,2,3]
15 g *Amomum subulatum* (fruit)[1,3]; *A. medium*[2]
15 g *Terminalia chebula* (fruit)[1,2,3]
17.5 g *Terminalia belerica* (fruit)[1,3]; *Melia toosendan*[2]
20 g *Emblica officinalis* (fruit)[1,3]; *Crataegus pentagyna*[2]
35 g *Tinopora cordifolia* (plant)[1,3]; *Sophora flavescens*[2]
7.5 g *Zingiber officinale* (root)[1]; *Hedychium spicatum*[2,3]
15.5 g *Solanum xanthocarpum* (stem)[1]; *Sambucus sibirica*[2]; *Rubus idaeopsis* [3]
22.5 g *Inula racemosa* (root)[1,3]; *Paeonia albiflora*[2]
12.5 g *Justicia adhatoda* (plant)[1,3]; *Odontites serotina*[2]
15 g *Swertia chirayita* (plant)[1,3]; *Gentiana barbata*[2]
17.5 g *Picrorhiza kurrooa* (root)[1,3]; *Scutellaria baicalensis*(root, stem, leaf)[2]
17.5 g *Shorea robusta* (exudate)[1,3]
17.5 g *Commiphora mukul* (exudate)[1,3]; *Vatica lanceaefolia*[2]
5 g musk[1,2,3]
17.5 g *Strychnos nux-vomica* (fruit)[1,3]
12.5 g *Mesua ferrea* (flower)[1,3]; *Quisqualis indica*[2]
12.5 g *Saussurea lappa* (root)[1,3]
22.5 g *Inula britannica* (plant)[1,2]; *Chrysanthemum tatsienens*[3]
22.5 g *Meconopsis horridula* (plant)[1,3]
7.5 g human flesh[1]; Kochela[2]; wild yak's heart[3]
25 g *Aconitum gymnondrum* (leaf)[1]; *A. napellus*[2]; *A. spicatum*[3]
22.5 g *Carduus crispus* (plant)[1,2]; *Pulicaria insignis*[3]
17.5 g *Canavalia gladiata* (fruit)[1]; *Senecio krylovii*[2]; *Melia composita* [3]
10 g *Punica granatum* (fruit)[1,2,3]
15 g *Stellaria dichotoma* (plant)[1,2]

Use and action: Heart disease[1]
Chronic fever[1,3]

Arthritis[1]
Dry cough[1]
Backache[2,3]
Difficulty in walking[2]
Difficulty in breathing[3]
Unlocalized pain[3]
Very safe and gentle drug for general rLung[3]

Dosage: 500 mg in the morning and evening with hot or warm water.

5) A ru bchu pa[1,2] 8 ingredients

Composition:
25 g *Terminalia chebula* (fruit)[1]
10 g *Crocus sativus* (flower)[1]
10 g *Elettaria cardamomum* (fruit)[1]
10 g bitumen[1]
10 g *Gentiana barbata* (plant)[1]
10 g *Canavalia gladiata* (fruit)[1]
10 g *Symplocos racemosa* (leaves)[1]; *Eriobotrya japonica*[2]
10 g *Rubia cordifolia* (leaves)[1]; *Galium boreale* (root)[2]
10 g vermillion[1] ; *Juniperus communis* (leaves)[2].

Use and action: Sores in the kidneys[2]
Swelling and obstruction in passing urine[2]
Pain in the waist[2]
Difficulty in walking[2]
Kidney fever[1,2]

Dosage: 500 mg in the morning.

6) Bi ma la i sbyor ba[1,2,3] 19 ingredients

Composition:
20 g *Myristica fragrans* (fruit)[1,2,3]
20 g *Terminalia chebula* (fruit)[1,2,3]
20 g *Shorea robusta* (exudate)[1,3]; *Boswellia carteri*[2]
20 g *Aquilaria agallocha* (stem)[1,3]; Black *Terminalia chebula*[2]
10 g solidified ox or elephant bile[1]; *Caryx* sp.[3]; *Hedychium spicatum*[2]
10 g *Ferula asa-foetida* (exudate)[1]; *F. jaeschkeana*[3]; *Caesalpinia sepiaria*[2]
10 g *Syzygium aromaticum* (flower)[1,2,3]
10 g *Bambusa arundinacea* (exudate)[1,2,3]
10 g *Elettaria cardamomum* (fruit)[1,3]; *Melia toosendan*[2]
10 g *Amomum subulatum* (fruit)[1,3]; *A. medium*[2]
10 g *Carum carvi* (fruit)[1,3]; *Anisum vulgare*[2]
10 g *Santalum album* (stem)[1,2,3]
10 g *Pterocarpus santalinus* (stem)[1,2,3]
10 g *Terminalia belerica* (fruit)[1,3]; *Crataegus pinnatfida*[2]
10 g *Emblica officinalis* (fruit)[1,3]; *Rhamnus dahuricus*[2]
10 g *Canavalia gladiata* (fruit)[1]; *Melia composita*[3]; *Sterculia alata*[2]
10 g *Acacia catechu* (stem)[1]; *Crocus sativus*[2]; *Carthamus tinctorus*[3]
10 g *Allium sativum* (root)[1,2,3]
10 g *Cymbopagan martini* (root)[1]; *Bergenia crassifolia*[2]; *Geranium* sp.[3]

Use and action: Heart diseases [1,2]
Discomfort in cardiac region[2,3]
Lack of concentration and forgetfulness[2,3]
Mental disorders and epilepsy [1,2]
Chest and upper back pain[3]
Despondency, fatigue, nervousness[2,3]
Cerebral ischemia[3]

Dosage: 2-3 g twice daily in the morning or night with hot water.

7) **'Bras-bu gsum-thang**[3] 3 ingredients

Composition:
Terminalia chebula
Terminalia belerica
Emblica officinalis

Use and action: Febrifuge
Blood purifier
Balances blood and *rLung*
Chronic fever and fatigue

Dosage: 3-5 g twice daily with a decoction boiled down to 1/3 level.

8) **Ga bur ñi śu rts lna**[1,3] 25 ingredients

Composition:
3 g *Cinnamomum camphora* (exudate)[1,3]
10 g *Bambusa arundinacea* (exudate)[1,3]
3.5 g *Crocus sativus* (flower)[1,3]
4 g *Syzygium aromaticum* (fruit)[1,3]
6 g *Myristica fragrans* (fruit)[1,3]
8.5 g *Elettaria cardamomum* (fruit)[1,3]
8.5 g *Amomum subulatum* (fruit)[1,3]
5 g *Aquilaria agallocha* (stem)[1,3]
9 g *Santalum album* (stem)[1,3]
6.5 g *Pterocarpus santalinus* (stem)[1,3]
5.5 g *Nelumblo nucifera* (flower)[1]; *Meconopsis grandis*[3]
11 g *Nelumbo nucifera* (pistil)[1]; *Foeniculum vulgare*[3]
11 g *Mesua ferrea* (flower)[1,3]
9 g *Saussurea lappa* (root)[1,3]
9 g *Cuminum cyminum* (fruit)[1]; *Glycyrrhiza glabra*[1]
5 g *Aristolochia griffithi* (plant)[1]; *A. moupinensis*[3]
9 g *Cinnamomum zeylanicum* (stem bark)[1]
6.5 g Chu-srin sDer-mo (*Selaginella pulvinata*?)[1,3]
6.5 g *Nardostachys jatamansi* (plant)[1,3]
8 g *Dendrobium moniforme* (root)[1]
14 g Accumulated soil on rocks [1,3]
5 g *Hydrocotyle nepalensis* (plant)[1,3]
9 g *Terminalia chebula* (fruit)[1,3]
5 g *Terminalia belerica* (fruit)[1,3]
6 g *Emblica officinalis* (fruit)[1,3]
Rock sugar[3]

Use and action: All types of fevers affecting heart, liver, spleen, kidney and lungs[1,3]
Malaria fever[1]
Rheumatic arthritis[1,3], gout[3]
Abscess and septicemia[1]

Dosage: 250 mg twice daily at noon and night in hot water[1]
2-3 g once daily in the morning with hot water[3]

9) Gser-mdog-bchu-gsum[2] 13 ingredients

Composition:

Piper longum
Crocus sativus
Lilum sp.
Sophora flavescens
Olea europea
Justicia ganderussa
Costus speciosus
Salt
Cinnamomum camphora
Soma plant (*Herpetospermum caudgerum* ?)
Rhododendron sp.
Iron filings
Snake meat

Use and action: high blood pressure
jaundice
tumours
indigestion
stomach trouble
fever

Dosage: not specified

10) Gser-mdog-bchu-pa[2] 13 ingredients

Composition:

Soma plant (*Herpetospermum caudgerum* ?)
Terminalia chebula
Glycyrrhiza glabra
Bitumen
Zingiber officinale
Punica granatum
Elletaria cardaomum
Piper longum
Erycibe paniculata
Rock salt from Sindh in Western India

Use and action: Diptheria
High blood pressure

Dosage: One spoonful daily to be taken with hot water

11) Gur gum bdun pa[1,2] 7 ingredients

Composition:

50 g *Crocus sativus* (flower)[1,2]
40 g *Bambusa arundinaceae* (exudate)[1,2]
10 g Solidified ox or elephant's bile[1,2]
50 g *Nelumbo nucifera* (plant)[1]; *Myristica fragrans* (fruit)[2]
50 g *Justcia ahdatoda* (plant)[1]; *Aquilaria agallocha* (stem)[2]
50 g *Callicarpa macrophylla* (plant)[1] ; *Sterculia alata* (bark)[2]
25 g Bitumen[1]; *Inula helenium* (root)[2]

Use and action: Heart fever[2]
Liver fever[1]

Dosage: Three spoonful mixed and boiled in a pint of water until water is reduced to two-thirds[2]
500 mg in the morning with hot water[1]

12) Nor bu bdun than[1,2,3] 7 ingredients

Composition:

150 g *Terminalia chebula* (fruit)[1,2,3]
150 g *Terminalia belerica* (fruit)[1,2,3]
150 g *Emblica officinalis*[1,3]; *Olea europea*[2]
150 g *Inula helenium* (root)[1,2]; *Saussurea lappa*[3]
150 g *Tinospora cordifolia* (plant)[1,3]; *Sophor flavescens*[2]
300 g *Solanum xanthocarpus* (stem)[1]; *S. jaquina*[2]; *Rubus idaeopsis*[3]
100 g *Zingiber officinale* (root)[1]; *Hedychium spicatum*[2,3]

Use and action: All diseases of *khrag* and *rLung*[1]
Clears up fever due to inflammation of the blood[3]
High blood pressure[2]
Fever[2,3]
Colds[2,3], influenza[2]

Dosage: One cup of decoction (ground plants in 4X boiling water, reduced to 1/4 and strained), morning and afternoon[1]
Decoct 3-5 grams to 1/3rd water level and take once daily

13) Rje'u gtso bo brgyad pa[2] 8 ingredients

Composition:

100 g Solidified ox or elephant bile
100 g *Santalum album* (stem)
100 g *Bambusa arundinaceae* (exudate)
100 g *Crocus sativus* (flower)
100 g *Swertia chirayita* (plant)
100 g *Justicia adhatoda* (plant)
100 g *Picrorhiza kurrea* (root)
100 g *Aconitum tanguticum* (plant)

Use and action: Fever and diseases of the heart and liver

Dosage: 250 mg twice daily, morning and evening in hot water

14) Se 'bru lna pa[1,2,3] 5 ingredients

Composition:

40 g *Punica granatum* (fruit)[1,2,3]
2.5 g *Cinnamomum zeylanicum* (stem bark)[1,3]; *C. cassia*[2]
2.5 g *Elettaria cardamomum* (fruit)[1,2,3]
5 g *Piper longum* (fruit)[1,2,3]
20 g *Alpinia officinarum* (fruit)[1]; *Hedychium spicatum*[2,3]
Molasses[2]

Use and action: Cardiac pain[1]
Air in the heart[2]

Indigestion, stomach disorder[1,2,3]
Pain in kidney and lumbar region[1,3]
Kidney disease[2]

Dosage: 500 mg, morning and evening in very hot water[1]
2-3 g once daily with hot water[3]

15) Se 'bru bryad pa[2] 5 ingredients

Composition:

Punica granatum
Cinnamomum cassia blume
Elettaria cardamomum
Piper longum
Myristica fragrans
Crocus sativus
Amomum medium
Hedychium spicatum

Use and action: Sstomach diseases
Liver diseases
Phlegm and air diseases
Heart tumour

Dosage: not specified

16) Sho sha ai sman mar[2] 5 ingredients

Composition:

Aquillaria agallocha
Santalum album
Areca catechu
Sterculia alata
Vitis vinifera

Use and action: Heart fever

Dosage: Boil sugar in water until water evaporates, then mix in the powdered ingredients and make into pills

17) Skyu-ru ñer lna[1,3] 25 ingredients

Composition:

200 g *Emblica officinalis* (fruit)[1,3]
100 g *Justicia adhotoda* (plant)[1]; *Veronica ciliata*[3]
50 g *Aristolochia griffithi* (plant)[1]; *A. moupinensis*[3]
50 g *Callicarpa macrophylla* (plant)[1]; *Meconopsis grandis*[3]
100 g *Coriandrum sativum* (fruit)[1,3]
50 g *Picrorhiza kurrea* (plant)[1,3]
25 g Bitumen[1,3]
25 g *Nymphae stellata* (plant)[1]; *Dracocephalum tanguticum*[3]
50 g *Pterocephalus hookeri* (plant)
50 g *Crocus sativus* (flower)
25 g Solidified ox or elephant bile
50 g *Pterocarpus santalinus* (stem)
50 g *Rubia cordifolia* (plant)
50 g Red lac
15 g *Nardostachys jatamansi* (plant)
25 g *Onosoma* sp. (root)
50 g *Cymbopogan martini* (root)
25 g *Swertia chirayita* (plant)

50 g *Terminalia chebula* (fruit)
2.5 g *Michelia champaca* (fruit)
25 g *Saussurea lappa* (root)
25 g *Inula racemosa* (root)
25 g *Solanum xanthocarpus* (stem)
50 g *Tinospora cordifolia* (plant)
25 g *Gacinia penduculata* (fruit)

Use and action: Blood diseases
Purifies blood

Dosage: Two pills of 250 mg each morning, noon and evening with hot water.