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ANTIOXIDANTS

IN

CANADIAN BOREAL FOREST

INDIGENOUS MEDICINAL PLANT TREATMENTS

IN RELATION TO

NON-INSULIN DEPENDENT DIABETES MELLITUS

by

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Figure 1. A representation of the boreal forest

ABSTRACT

Medicinal plants, as part of traditional ingestion practices, may contain antioxidants to combat the oxidative stress which is implicated in prediabetes as well as many of the complications of diabetes. As Indigenous Peoples move further from their traditional lifestyles, and therefore their use of medicinal plants, incidence of diabetes has increased dramatically. Those medicinal plants of the boreal forest that have been used for 3 or more symptoms of diabetes or its complications were selected for analysis. Three different assays (DPPH, NBT/xanthine oxidase and DCF/APPH) determined the antioxidant activity of 35 medicinal plant species. The majority of the species (89%) had free radical scavenging activity significantly greater than the market produce tested (Tukey, $P<0.05$), 63% had superoxide scavenging activities similar to vitamin C, and eight species had free radical scavenging activity similar to green tea. Considering that many of these species are also used for food or beverage they represent an antioxidant benefit to the traditional lifestyle. Among the parts used medicinally, roots and barks were used the most frequently with activity in the order of fruit>bark>leaves>roots. The perennials selected had activity in rank trees>shrubs>herbs and the activity associated with habitat found rocky areas>woodland>wet/boggy habitats. Species used for symptoms such as diarrhea, rheumatism, tonic and heart/chest pain were typically high in antioxidant activity. Using cluster analysis it was determined that species used for diarrhea and heart disease as well as those used for a combination of tonic, sores, urinary, blood, pregnancy and boils could also be species with high antioxidant activity. The greater the number of symptoms a species was used for, the greater the activity. Three species with high antioxidant activities, *Rhus hirta*, *Cornus stolonifera* and *Solidago canadensis*, inhibited TNF production in human macrophage cells suggesting a possible role in decreasing insulin resistance. The conclusions based on habitat, collection practices and symptom analysis give insights into methods for identifying antioxidant plant remedies. These results support the use of these traditional medicines in a lifestyle historically low in the incidence of diabetes and verify the selection and preparation of these plant remedies in relation to antioxidant activity.

RESUMÉ

Les plantes médicinales, faisant partie de le mode de vie traditionel, peuvent contenir des antioxydants pour combattre le stress oxydatif impliqué dans le prédiabète, ainsi que les complications du diabète. L'incidence du diabète parmi les peuples autochtones augmente dramatiquement lorsque ceux-ci s'éloignent de leur mode de vie traditionel. Des plantes médicinales de la forêt boréale ont été sélectionnées selon leur utilisation dans le traitement traditionel d'au moins trois symptômes du diabète ou de ses complications. Trois essais différents (DPPH, NBT/xanthine oxydase, et DCF/AAPH) ont déterminée l'activité antioxydante de 35 espèces de plantes médicinales. La majorité de ces espèces (89%) on démontré une activité significativement supérieure aux produits commerciaux testés (Tukey, $P<0.05$), 63% ont démontré une activité égale au vitamine C, et huit espèces était comparable au thé vert. Les racines et les écorces étaient utilisées le plus souvent, et que les parties médicinales des plantes avec la plus haute activité étaient le fruit>l'écorce>la feuille>la racine. Des plantes vivaces ont été sélectionnées selon leur activités antioxydante parmis les arbres>arbustres>herbes, et parmis celles qui poussent dans les milieux rocheux>boisé>marécageux/tourbeux. Les espèces utilisées pour combattre la diarrhée, le rhumatisme, les maux de coeur, ou comme tonifiant, ainsi que celles utilisées pour traiter un plus grand nombre de symptômes, démontraient typiquement une haute activité antioxydante. Une analyse typologique a déterminé que les plantes utilisées pour traiter la diarrhée, et les maladies cardiovasculaires, et aussi les combinaison de plais, furoncles, maladies urinaires et sanguinaires, pour faciliter la grossesse, et comme tonifiant, peuvent être également des espèces fortes en activité antioxydante. Trois espèces, *Rhus hirta*, *Cornus stolonifera* et *Solidago canadensis* avaient les plus hautes activités antioxydantes, et ont démontré une capacité d'empêcher la production de TNF dans les macrophages humains, ce qui suggère un rôle dans la diminution de la résistance à l'insuline. Nos résultats supportent l'utilisation de ces plantes traditionelles dans une mode de vie où l'incidence du diabète était historiquement basse. Nos conclusions apportent un support scientifique au mode la sélection et de préparation de ces plantes en relation avec leurs activités antioxydantes.

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CONTRIBUTIONS OF AUTHORS

As this is a manuscript-based thesis additional requirements include connecting texts between the manuscripts, one comprehensive reference list, a final conclusion and summary, additional materials (e.g.. appendices) where appropriate, and a statement in regard to the contribution of co-authors (i.e. contributions of authors).

All of the manuscripts of this thesis are co-authored by Dr. Timothy Johns who provided a supervisory role that included advice on the objectives, data analysis and presentation of results, editing and securement of funding for the laboratory procedures. All other aspects were the responsibility of the primary author, Letitia McCune.

CONTRIBUTIONS TO KNOWLEDGE/ STATEMENT OF ORIGINALITY

Despite the growing body of scientific and popular literature on antioxidants there have been few works on the contributions of antioxidants to traditional lifestyles outside the analysis of antioxidant vitamins in traditional foods. Presented here is the first report on the contribution of antioxidant activity by a set of medicinal plant species used by the Indigenous Peoples of the boreal forest. Although the doses and average rates of consumption are unknown, this study adds to the knowledge of general antioxidant potential of a traditional lifestyle. Subsequent analyses of these and other medicinal plants would give a clear picture of overall antioxidant status of a traditional lifestyle as a combination of food and medicinal plant contributions.

Traditional plant remedies for diabetes are common throughout most of the world. With the increased incidence of diabetes worldwide, especially among the indigenous populations, the search for diabetic treatments has led to the analysis of some of these plants for hypoglycemic activity. There has also been an increase in knowledge regarding the influence of oxidative stress in diabetes and its complications. This is the first study to document that the antioxidants in plant treatments may be another possible mode of action for plants used for symptoms of diabetes and its complications. This study may then lead the way for other studies into the antioxidant benefits of plants used worldwide for diabetes.

To the best of our knowledge native Canadian plant species have not been scrutinized for hypoglycemic activity, most likely because few of the species are specifically recommended for diabetes. Presented here is a list of 35 plant species selected for their use for a combination of symptoms associated with diabetes. This is the first such list of Canadian plant species. This list may lead to further analyses of these particular plant species for potential treatments of diabetes.

In analyzing the antioxidant activities of these species in relation to specific symptoms it was determined that certain symptoms are better predictors of antioxidant activity than others. In addition, the work of this thesis has demonstrated that certain groups of

symptoms can also be predictors of high antioxidant activity. The number of symptoms a plant species is used for was also found to be significantly related to antioxidant activity. There have been no other reports of this nature, especially in regard to symptoms of diabetes. In any subsequent searches of plant treatments for potential antioxidant activity this information will be of benefit.

From analysis of different plant tissues within a species of these medicinal plants insights have been drawn into the selection practices of herbal gatherers of plant material. Antioxidant activities were higher than expected in all of the plant tissues used medicinally when compared to all tissues in the same set of plant species- with the exception of roots. These observations emphasize the importance of following the gathering practices of the traditional herbalist. Collection of the correct part of a species can be as important as the identification of the species. This thesis therefore has contributed to the scientific validation of the traditional gathering practices. This is important when considering the benefit of different levels of antioxidants for different medical conditions.

Plant structure and habitat condition were also found to be significantly correlated to antioxidant activity. Antioxidant differences found among the categories of habitat and structure may lead to further physiological studies, or studies at the genetic level, in regard to the oxidative stress encountered by species growing under similar circumstances. These observations also suggest what type of plant to search for, or what habitat to look in when searching for plants of high antioxidant activity in the boreal forest.

In our analysis of antioxidant activity a compilation of antioxidant activity was generated for 35 different plant species. The activity was determined for free radical scavenging as well as superoxide and peroxyl radical scavenging *in vitro*. In and of itself this represents a contribution to knowledge in regard to each of these species. In addition the comparative analysis identified the top antioxidant species for possible further medical applications.

Finally, in analyzing the top selected plant species in antioxidant activity for the ability to inhibit the production of tumor necrosis factor this study has opened up the possibility of

other plants used for diabetes to be similarly tested in order to determine their potential affect on insulin resistance. This is the first report testing plant extracts in this system as a means of determining potential antidiabetic effects. In addition, in reporting the viability of the macrophage and the inhibition at various doses of each of the three species, there has also been a contribution to knowledge in regard to each of these species.

In summary this thesis has contributed significantly to the scientific validation of traditional plant medicinal practices of the Indigenous Peoples of the boreal forest. It has added to the knowledge base in regard to the antioxidant activities of 35 different plant species and will hopefully encourage further work on the antioxidant contributions of plant treatments for diabetes.

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LIST OF ABBREVIATIONS

AAPH:	2,2'-diazobis(2-aminopropane)dihydrochloride, an azo initiator of peroxyl radicals
AMVN:	2,2'-azobis(2,4-dimethylvaleronitrile), a lipophilic azo initiator of peroxyl radicals
BB	biobreeding/Winstar diabetic mouse
CBD	Convention of Biological Diversity
CINE	Centre of Indigenous Peoples' Nutrition and Environment
Cs:	Cornus stolonifera
DCF:	2,7-dichlorofluorescin-diacetate The abbreviation of the assay using the above in combination with AAPH to monitor peroxyl radical scavenging.
DHAA	dehydroascorbate
DPPH:	1,1 diphenyl-2-picryl-hydroazyl, a hydrogen donator The abbreviation of the assay using the above for the analysis of free radical scavenging.
GSH	reduced glutathione
GSSG	oxidized glutathione
HPLC	high-performance liquid chromatography
IDDM:	Insulin dependent diabetes mellitus, also known as type 1 diabetes or juvenile diabetes
LDL	low-density lipoprotein
MLDS	multiple-low-dose streptozotocin
NBT:	Nitroblue tetrazolium, used to analyze the XO assay spectrophotometrically
NAD	nicotinamide adenine dinucleotide
NADH	the reduced form of NAD
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	the reduced form of NADP

NIDDM:	Non-insulin dependent diabetes mellitus, type 2 diabetes or adult onset diabetes
NOD	nonobese diabetic mouse
PC	Phosphatidylcholine
ppm	parts per million (μg/g of liquid)
ROS	Reactive oxygen species
SOD	Superoxide dismutase
STZ	streptozotocin
Rt:	Rhus hirta
Sc:	*Solidago canadensis*
TNF:	Tumor necrosis factor-α
TBA	thiobarbituric acid also another name for the TBARS assay
TBARS	an assay using thiobarbituric acid reactive substances
TCA	thiocyanate assay
XO:	Xanthine oxidase
	The abbreviation of the assay using a hypoxanthine/xanthine oxidase generating system for the analysis of superoxide radical scavenging.

INTRODUCTION

The World Health Organization estimates that by the year 2010 diabetes will affect 221 million people worldwide (Amos *et al.*, 1997). Indigenous Peoples in Canada have an alarmingly greater prevalence of diabetes as compared to all Canadians (Young,1994). This is especially noticeable as their lifestyle changes from traditional hunter-gatherer to sedentary market consumer. This lifestyle change includes a decreased use of traditional plant medicines.

Traditional plant medicines are used by 90% of the people in developing countries as the main form of medical intervention. There are many traditional remedies associated with diabetes. Marles and Farnsworth (1995) reviewed 1200 species used worldwide for diabetes and found 81% of 295 species were reported to be active in hypoglycemic tests. Traditional plant remedies, however, may affect diabetes in ways other than lowering blood glucose.

In diabetes oxidative stress is known to increase throughout the body causing increases in lipid peroxidation and glycoxidation products causing, among others, a decrease in arterial wall function (Baynes, 1991). In fact oxygen radicals have been associated with many human clinical conditions including some considered to be complications of diabetes mellitus (Halliwell and Gutteridge, 1990). Plants, and therefore also plant medicines, can accumulate numerous antioxidants including antioxidant enzymes, ascorbic acid, α-tocopherol and phenolics including flavonoids (Pratt, 1992). These antioxidants in plant medicines could therefore decrease the oxidative stress, and symptoms, associated with aspects of diabetes etiology.

It has been suggested that Indigenous People are genetically prone to diabetes. There is considerable literature revolving around this issue beginning with the idea of a "thrifty gene" which once allowed rapid conversion of feasts to fat for use in times of famine (Neel, 1962; Diamond,1992; Sakul *et al.*,1997). If the Indigenous Peoples of Canada are genetically

prone to diabetes, it is therefore possible that their traditional medicinal plants contain treatments for the symptoms of diabetes and/or the complications of diabetes. Canadian indigenous medicinal plants have not been screened for hypoglycemic activity or antioxidant activity. Antioxidants are found in plants and those medicinal plants selected to treat the symptoms of diabetes and its complications may have higher levels. Research into these medicinal plants could lead to a Western scientific understanding of the traditional indigenous lifestyle as well as insights into the treatment of diabetes.

The hypothesis of this research proposal is that there are antioxidants in abundance in the Canadian indigenous medicinal plants used to treat diabetic symptoms. The general objectives include:

1. Determine if there were plants used in the study area (the boreal forest of Canada) for the symptoms of diabetes or its complications.
2. Analyze those plants used for diabetic symptoms and its complications for various types of antioxidant activity.
3. Determine if the antioxidant activity is related to a particular symptom or set of symptoms.
4. Analyze various methods of plant preparation for antioxidant activity.
5. Characterize some of the selected plants for activity in relation to plant tissue type, growth structure and habitat.
6. Determine, from at least a few of the plants highest in antioxidant activity, if there is also a direct action to reduce the instigation of non-insulin dependent diabetes mellitus (NIDDM).

Fulfilling these objectives may emphasize the need to preserve the indigenous knowledge and use of these medicinal plants as well as contribute to potential antioxidant therapies for those with NIDDM. In addition the results could increase the recognition of the ecological knowledge of these people and the importance of their traditional gathering methods. This research is fundamental to our understanding of strategies to decrease this prominent disease not only for all of humankind but for this particularly prone population.

LITERATURE REVIEW

Diabetes and the Indigenous People

Diabetes

Diabetes mellitus is a disease of high blood glucose. The two most common types of diabetes mellitus are type 1 representing insulin dependent diabetes mellitus (IDDM) and type 2 or non-insulin dependent diabetes mellitus (NIDDM). IDDM represents 10-15% and NIDDM 80-90% of the cases of diabetes. Another type of diabetes is gestational diabetes mellitus which represents 1-3% of the total cases, 30% of these later develop NIDDM (Oberley,1988).

IDDM is also termed juvenile onset diabetes since it occurs in individuals below the age of 30 when an autoimmune destruction of the beta cells of the pancreas stops the production of insulin. Symptoms include polyuria, polydipsia, glucosuria, fatigue and weakness. As the disease progresses symptoms can include blurred vision and ketoacidosis.

NIDDM usually occurs after the age of 30 (adult onset) and often, but not always, in obese individuals. The beta cells are intact in NIDDM but there is a resistance in target tissues to insulin causing an abnormal amount of insulin secretion from the pancreas. Symptoms include polyuria, polydipsea, glucosuria, fatigue and gradual weakness. There is no ketoacidosis.

The later complications in both types of diabetes include renal failure, retinopathy and cataracts, coronary heart and artery disease, peripheral arteriosclerotic disease and peripheral neuropathies (impotence, numb/painful feet, footsores/ulcers) and fungal and bacterial infections (Carlson, personal communication). Some of these complications are known to involve oxygen radicals. Since atherosclerotic vascular disease is the leading cause of

mortality in those with diabetes (Giugliano *et al*, 1995), much of the current research focuses on the oxidative stress connected with diabetes and vascular complications.

Environmental and Genetic Effects

Canadian Indigenous Peoples have a much greater prevalence of NIDDM than the Canadian average. In 1991 the prevalence was 6% among the total indigenous population compared to the all Canadian average of 2.5% (Young, 1994). The numbers can increase to over 10% depending on gender and region of the country. Age can also make a difference as illustrated by the prevalence of NIDDM at 20% for American Indigenous men (versus 10% for white men) and 28% for American Indigenous women (versus 12% for white women). The region of the country can also affect the NIDDM rate via changes in lifestyle. There is an increase in diabetes as people become more urbanized and the diet changes composition. Obesity has been shown to increase with the consumption of market foods and decreased physical activity. This is illustrated by the American Indigenous Peoples overweight rates of 34% for American Indigenous men (24% for all U.S. men) and 40% for American Indigenous women (25% for all U.S. women)(Young, 1994). Prevalence of obesity and diabetes is increased in women and increases with age.

It is thought that in concert with the environmental factors are genetic factors predisposing Indigenous Peoples to diabetes. The theory of the "thrifty genotype" (Neel , 1962) suggests a genotype quick to convert glucose to fat to increase survival in times of famine. Diamond (1992) expanded on this concept in reviewing studies of a diabetes epidemic on Nauru Island in the Pacific. He emphasizes that fast "coco-colonization" (i.e. the drastic change of lifestyle of indigenous populations that can occur as illustrated in the colonization of areas for cocoa or coffee production) of Indigenous Peoples has led to a greater incidence of diabetes as compared to Europeans that have had the action of natural selection on its population over time (before the advent of insulin). Sakul *et al.*(1997) culminated a series of projects by C. Bogardus and colleagues on the genotypes of the Pima Indians and found that the variance in insulin action in this group is 38-49% familial.

A genetic predisposition to NIDDM may exist in a defect in the beta cell's ability to respond to increased insulin resistance. After hyperinsulinemia is established the beta cells become exhausted. Hyperinsulinemia has been shown to lead to increased insulin resistance due to a decrease in insulin receptors in the target tissues and postbinding defects in the metabolism of glucose (Beebe *et al.*, 1991). The increased insulin resistance leads to a group of symptoms termed "syndrome X" which leads to coronary heart disease. These include increased plasma triglycerides, decreased high density cholesterol and increased blood pressure (Reaven, 1995). Many of these symptoms have been associated with oxidative stress with corresponding research involving antioxidants such as vitamin E (Kunisaki *et al.*,1993; Reaven *et al.*,1995; Rosen *et al.*,1995; Keegan, 1995).

Conventional and Traditional Treatments for Diabetes

Currently the method of treatment of NIDDM includes diet and weight loss to decrease hyperglycemia. Blood glucose levels are maintained via strict dietary monitoring and the use of the "exchange system" for diet planning (Zeman and Hanssen,1991). In the reduction of obesity even a slight amount of weight loss can stimulate insulin action (Beebe *et al.*, 1991). In addition exercise is suggested since it is known to increase insulin sensitivity and improve glucose tolerance.

If these methods fail to achieve the desired result, hypoglycemic agents are given. These include sulfonylureas to increase insulin release, biguanides to decrease hepatic gluconeogensis and thiozolidine to increase insulin receptor kinase. If these also fail then insulin is given by injection.

Interestingly some of the same type of hypoglycemic compounds can be found in plants. There is a high amount of guanides in *Galega officinalis*, or goat's rue, and hypoglycemic sulfur-containing compounds are present in onion and garlic (allicine and allyl sulfide) as well as *Catharanthus* plants (leurosine sulfate)(Perl, 1988). Numerous plants have been screened for hypoglycemic activity. There have been several good reviews published on these plants including those by Marles and Farnsworth (1995) and Bailey and Day (1989).

Marles and Farnsworth review 1200 species while Bailey and Day cover 400. Many of these plants come from India or the orient and many have demonstrated activity. Little if any work has been done on native Canadian plants.

On a worldwide basis, some of the more widely used plants for the traditional treatment of diabetes include *Momordica charantia, Catharanthus roseus, Anacardium occidentale* and *Trigonella foenum-graecum.* Some active principles of these and many other plants are listed in Marles and Farnsworth (1995). *Momordica charantia*, also known as bitter gourd or balsam pear, is used worldwide. Its hypoglycemic principles include peptides, terpenoids and steroid glycosides that are involved in glucose uptake in muscle and glycogen accumulation. *Catharanthus roseus*, also known as periwinkle or *Vinca rosea*, acts as an insulin substitute and has 55 active alkoloids. *Trigonella foenum-graecum*, or fenugreek, is widely recognized as a folk remedy. It is used in curries and chutneys and has a number of hypoglycemic principles including a high fiber content which slows digestion (Bailey and Day, 1989, Marles and Farnsworth, 1995).

The hypoglycemic activity of any antidiabetic plant could be through a number of mechanisms including the reduction of enzyme action by the salivary glands, increased dietary fiber or inhibitors of digestion, stimulators of insulin action or secretion, insulin mimics or inhibitors of gluconeogenesis (Perl, 1988). Nutrient availability and rate of digestion are the main means of decreasing hyperglycemia through food and beverage sources (Johns and Chapman, 1994).

An overlap exists in many of the different biosynthetic groupings in which hypoglycemic agents and antioxidants belong. Some of these classes include tannins, flavonoids, polyphenols, coumarins and vitamins (Marles and Farnsworth, 1995). In the search for antioxidants in plants affecting diabetes it is possible that some hypoglycemic activity may also be found in specific samples. However although antioxidants, in and of themselves, have been found to affect the numerous types of oxidative stress in the diabetic they have not been found to conclusively lower glucose levels.

Because the number of diabetics among the indigenous communities has been steadily increasing it is largely viewed as a disease of the European considering incidence has increased with length of contact (Young, 1994). This contact contributed to the incorporation of market foods into the diet and a sedentary lifestyle. No references were found on medicinal plant treatments specifically for 'diabetes' among these populations. The approach by indigenous communities and organizations concerned with diabetes is to decrease the incidence of obesity and focus on nutrition education (e.g.. Kahnawake Schools Diabetes Prevention Project). Although various health programs (e.g.. Aboriginal Diabetes Wellness Center in Edmonton) incorporate traditional practices and encourage the use of traditional foods, there is largely no focus on traditional medicinal plants to help combat the effects of diabetes. It is suggested in this thesis that in addition to the above programs incorporation of some traditional medicinal plants would help to decrease the incidence of diabetes and its complications.

Oxidative Stress and Diabetes

Oxidative Stress

Antioxidants combat various forms of oxidative stress. Oxidative stress is a result of toxic reactive oxygen species (ROS). These typically include singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2) and peroxyl radical ($ROO^{\cdot}$). The reactivity, and therefore the destructive nature, of the radicals is due to an unpaired electron. Figure 2 illustrates the spin directions of the unpaired electrons as well as the production of some of the known radicals formed from oxygen. The hydroxyl radical is the most damaging causing lipid peroxidation as well as protein, enzyme and DNA damage. Hydrogen peroxide and lipid peroxides cause damage by producing further oxygen metabolites (including the damaging hydroxyl radical) in the presence of metal catalysts (e.g., the Fenton reactions).

Lipid peroxidation:

Initiation

$$LH \xrightarrow{H\cdot} L\cdot$$

Propagation of lipid radical (L·) and lipid peroxyl radical (LOO·)

$$L\cdot + O_2 \longrightarrow LOO\cdot$$
$$LOO\cdot + LH \longrightarrow LOOH + L\cdot$$

Termination by antioxidant (AH)

$$LOO\cdot + AH \longrightarrow LOOH + A\cdot$$
$$L\cdot + AH \longrightarrow LH + A\cdot$$

Fenton net reaction:

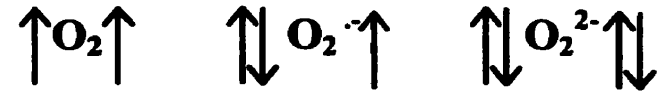
$$2H_2O_2 \longrightarrow O_2 + 2H_2O$$

from:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH\cdot + OH^-$$

$$Fe^{3+} + O_2^{\cdot-} \longrightarrow Fe^{2+} + O_2$$

Spin orientation of electrons in outer orbital:



Oxygen **Superoxide** **Peroxide**

Figure 2. Reactions and spin orientation related to various oxygen radicals (adapted from Halliwell,1989 and Cadenas,1995)

Most of the production of these ROS occurs in the mitochondria in animals and the chloroplasts in plants. Because the production of many of these ROS is inevitable with oxygen metabolism there are elaborate defenses in both animals and plants. In both systems there are endogenous enzymes acting as antioxidants to protect the organism. The superoxide dismutases (MnSOD, CuZnSOD) are key enzymes in the conversion of superoxide ions to hydrogen peroxide ($2\ 0_2^{\cdot-} + 2\ H^+ \rightarrow H_2O_2 + 0_2$). Catalase in the cytosol degrades hydrogen peroxide ($2\ H_2O_2 \rightarrow 2\ H_20 + 0_2$) and thereby reduces the components available for production of the damaging hydroxyl radical. The enzyme glutathione peroxidase scavenges lipid peroxides in both the mitochondria and cytoplasm [(L00H + 2 GSH(reduced glutathione) $\rightarrow$ LOH + H_20 + GSSG(oxidized glutathione)] (Thompson and Godin, 1995). Examples of antioxidants that are not enzymatic include ascorbic acid, alpha-tocopherol, glutathione and uric acid.

When free radicals are not scavenged lipid peroxidation can be initiated. Initiation is usually achieved via a hydroxyl radical, perhydroxyl radical ($HO_2^{\cdot}$) or a chelated iron-oxygen complex. Intermediate products include conjugated dienes, conjugated peroxy radicals and hydroperoxides. Termination results when there is a combination of two alkyl radicals, two peroxyl radicals or the combination of alkyl or peroxyl radicals with antioxidants. Lipid peroxidation occurs when cholesterol or phospholipids containing unsaturated fatty acyl moieties come under attack by these oxygen metabolites. Because membranes are composed of such susceptible lipids, lipid peroxidation is a primary cause of membrane damage. Such destruction can lead to disruption of membrane fluidity and the malfunction of enzymes located in the membranes. Several reviews have been published on oxidative stress as well as lipid peroxidation (Giriotti, 1985; Dix and Aikens,1993).

Oxidative Stress in the Diabetic

Once diabetes is established the whole body undergoes an increase in oxidative stress. The complications of diabetes are often related to oxygen free radicals (Figure 3). Baynes (1991) gives a good overview of oxidative stress and complications in diabetes. He emphasizes glycoxidation products (sequential glycation and oxidation reactions between reducing sugars and proteins) in the collagen of diabetics as a biomarker of oxidative stress.

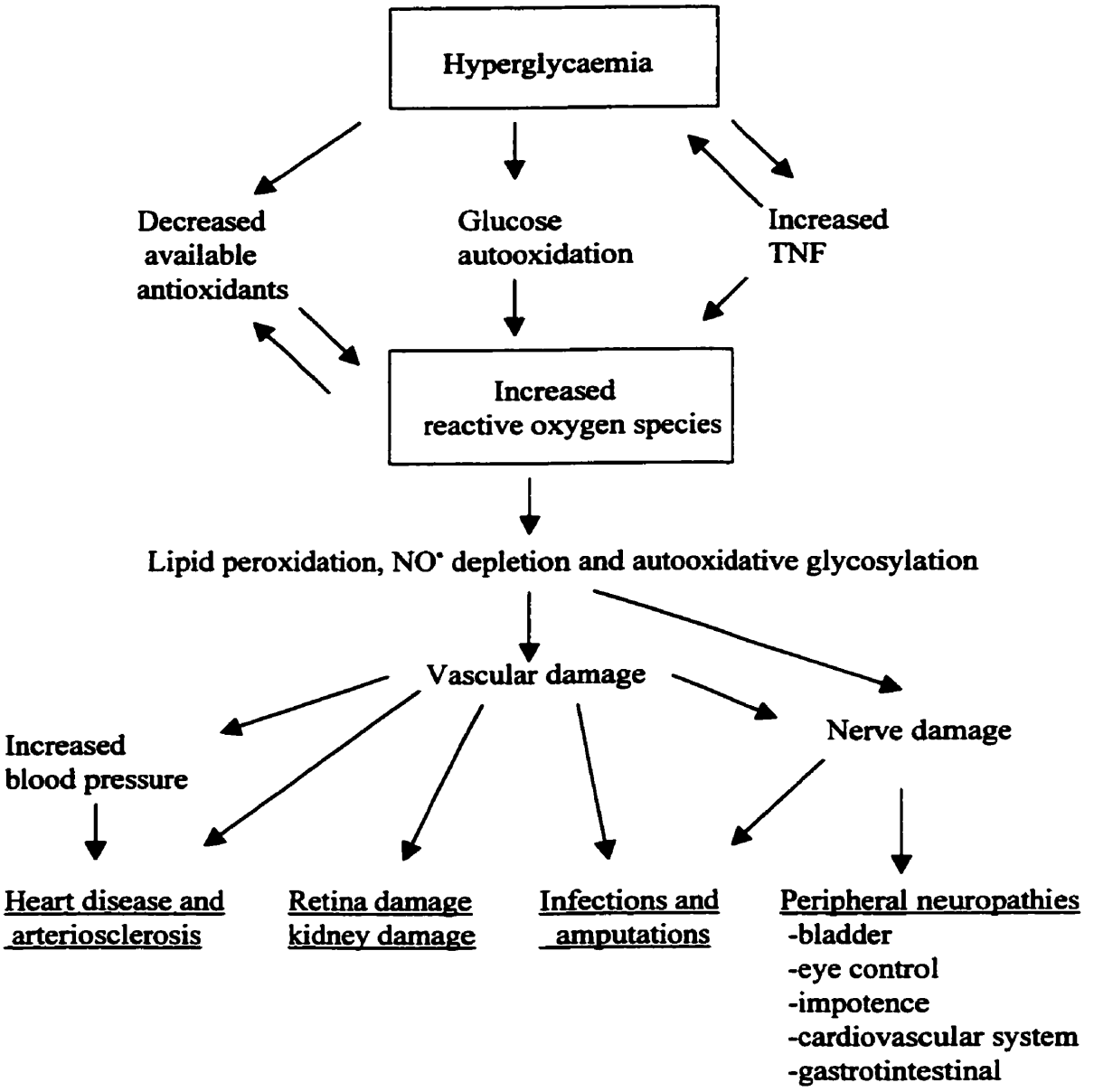


Figure 2. Overview of the effects of oxidative stress on the complications in NIDDM (adapted in part from Van Dam and Bravenboer,1997)

The increase in free radicals and the resultant protein damage can lead to a cycle of lipid peroxidation, cell death and scavenger enzyme malfunction that aggravates oxidative stress.

The liver, kidney, spleen, heart, testis, pancreas, skeletal muscle and erythrocytes of the diabetic have decreased total superoxide dismutase (SOD) activity (Oberley, 1988). Interestingly, type 1 has a different level of Cu-ZnSOD activity in erythrocytes compared to type 2 diabetes. Type 1 on insulin treatment showed no change in these levels while type 2 patients on hypoglycemic agents had a 97% reduction in activity in erythrocytes. This has been speculated to be due to an increase in the glucosylated form of Cu-ZnSOD which has a lower enzymatic activity (Oberley, 1988). Peroxidase activity increased in the liver, spleen, pancreas and skeletal muscle and the total glutathione content was lower in both the type 1 and type 2 human diabetic. The activity levels of glutathione peroxidase and reductase have been found to increase in type 2 diabetes (Oberley, 1988).

Kakkar *et al.* (1995) studied lipid peroxidase and antioxidant enzyme activity in rat tissues induced to be diabetic by 10 weeks on streptozotocin. Using the thiobarbituric acid reactive substances (TBARS) as a measurement of lipid peroxidation an increase was found in the heart, pancreas and blood. Lipid peroxidation results from oxygen free radicals that could be partly due to the autoxidation and protein glycation of glucose which is in high amounts in the diabetic. There were also increases in certain antioxidant enzymes which also suggests oxidative stress induction. Catalase activity increased in liver, heart and blood. Similar results were reported by Pieper *et al.* (1995) in 8 week streptozotocin induced rats. An increase in catalase was reversed with pancreatic islet transplants. Glutathione peroxidase also increased in Kakker's study in both the pancreas and kidney as well as superoxide dismutase in the liver, heart and pancreas. The initial low amount of antioxidant enzymes in heart and pancreas would make these organs more prone to lipid peroxidation damage.

Nourooz-Zadeh *et al.* (1995) argues against using TBARS as a measurement of lipid peroxidation in NIDDM patients, saying it is nonspecific and indirect. Using their new assay for hydroperoxides in blood plasma they reported a higher level in NIDDM patients

and an even higher level in those with diabetic complications compared to control subjects. In 1994, Chittar *et al.* also found higher levels of lipid peroxides in both NIDDM and IDDM human blood samples using a fluorometric method. They found their results varied depending on age, duration of diabetes, body mass index and ischemic heart disease.

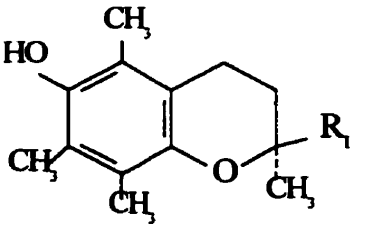
Glucose autoxidation and glycoxidation products can result in the decreased function of nitric oxide. This is important in vascular tone and endothelium-dependant relaxation and can result in arterial functional spasms (Giugliano *et al.*, 1995). Because the endogenous antioxidants are low in the blood of diabetics, such oxidative injury increases. Antioxidants were found to increase blood flow and decrease blood pressure and viscosity. Vitamin E in particular provides a decrease in nonenzymatic glycosylation, oxidative stress and red blood cell microviscosity in diabetics (Giugliano *et al.*,1995).

Antioxidant Treatments for Diabetes

Defense Mechanisms

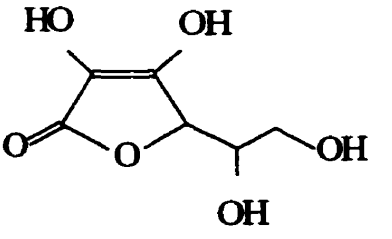
The aerobic life of animals requires various mechanisms for the removal of harmful oxygen-based radicals. These include enzymatic and nonenzymatic defenses that are frequently similar to those in plants. Both enzymatic and nonenzymatic defenses work in concert with each other (e.g., vitamin C may limit vitamin E reduction and vitamin E may save the use of glutathione). In animals the defense mechanisms include the nonenzymatic vitamin E, vitamin C, B-carotene (see figure 4), uric acid, bilirubin and carnosine as cellular protectors (Osawa, 1992). Estrogens have also been found to scavenge free radicals (Niki and Nakano, 1990). Interestingly, the bile pigment, bilirubin, acts as a chain-breaking antioxidant possibly protecting vitamin A and linoleic acid in the intestinal tract (Stocker *et al.*, 1987).

Enzymatic defenses include the iron-porphyrin peroxisome enzyme, catalase, which attacks hydrogen peroxides formed from oxidase reactions. Also present in the cytoplasm is copper-zinc superoxide dismutase which converts superoxide ions to hydrogen peroxide and bimolecular oxygen. In the mitochondria manganese superoxide dismutase and glutathione

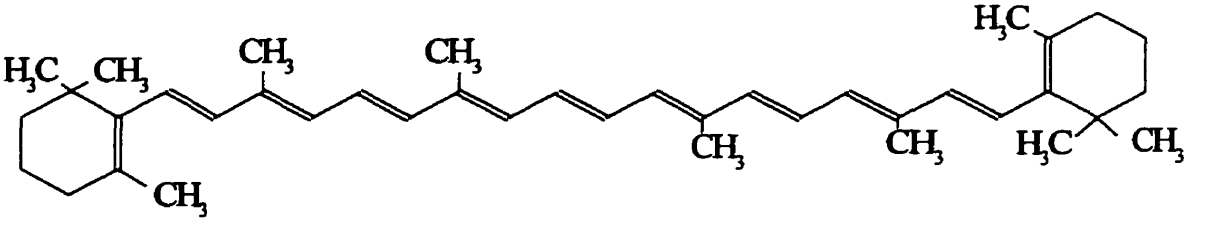


α-tocopherol

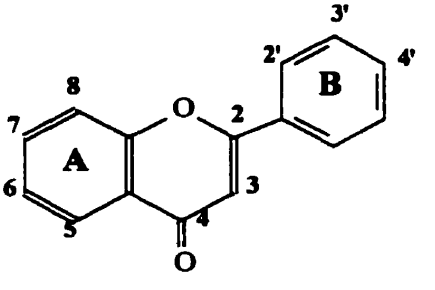
Trolox™



Ascorbic acid



β-Carotene



Flavone

Quercetin 3'=OH, 4'=OH,3=OH,5=OH,7=OH,

Figure 4. The structures of some common antioxidants

peroxidase are present. The selenoenzyme glutathione peroxidase can remove harmful hydrogen peroxides and its intermediates while phospholipid hydroperoxide glutathione peroxidase is able to detoxify cholesterol hydroperoxides. While antioxidants generally fall under the category of prevention of initiation of peroxidation (those that inactivate iron or scavenge oxyradical/hydrogen peroxides), they are also responsible for the enzymatic removal of hydroperoxide intermediates (Thomas *et al.*, 1990). The dismutases, catalases and glutathione peroxidase appear to have the highest activities in the liver and kidney most likely due to these organs' importance in detoxification and excretion (Grankvist *et al.*,1981).

Vitamin E

Vitamin E is the antioxidant most frequently studied in relation to diabetes. In the study of the initiation of diabetes there are two approaches: 1) studying vitamin E in relation to the destruction of the beta cells (IDDM) and 2) the effect on glucose control (NIDDM). Hayward *et al.* (1992) determined that in NOD (nonobese diabetic) mice (after up to 45 weeks of diet treatment) there is a protection of the beta cells by vitamin E. A previous study had also determined that the incidence of BB (biobreeding/Winstar) mouse diabetics could be reduced with vitamin E. They cite vitamin E's effect on immune response which includes the protection against the toxic oxygen radicals produced by inflammatory cells. They found, however, that insulinitis was not reduced.

Research has determined that vitamin E administered in pharmaceutical doses of 900mg per day for 4 months can increase the total body glucose disposal and increase the nonoxidative glucose metabolism in both control and NIDDM human patients (Paolisso *et al.*, 1993). The beta cell's response to glucose may be affected by the plasma ratio of oxidized glutathione to reduced glutathione considering that vitamin E is known to increase levels of glutathione. These results were contrary to those reported by Ozden *et al.* (1989) in streptozotocin rats given vitamin E by injection at 500-1000mg/kg for up to 21 days after the initiation of hyperglycemia. They found no improvement of glycemic control and a decrease in glycosylated hemoglobin. Reaven *et al.* (1995) also found no improvement in the glycemic indexes or reduction of protein glycation of vitamin E supplemented NIDDM humans (1600

IU/day for 10 weeks). These results however, could have been a factor of screening out those diabetics with unstable glucose levels.

Most of the current work focuses on vitamin E as an antioxidant and its function in various aspects associated with atherosclerotic vascular disease, the chief killer of diabetics. Previous work by Kunisaki *et al.* (1993) determined that there are specific binding sites for D-alpha tocopherol on cultured aortic endothelial cells. The low prevalence of vitamin E in those with vascular disease and diabetes has been correlated with a decrease in the platelet de-aggregator prostacylin produced by vascular endothelium cells. Reaven *et al.* (1995) concentrated their efforts on the effect of Vitamin E (1600IU/day for 10 weeks in NIDDM humans) on low-density lipoprotein (LDL) and protein glycation. They found that it did protect LDL from oxidation by enriching both the dense and buoyant LDL with vitamin E. Buoyant LDL had a greater capacity for enrichment than dense LDL's. These results are significant to NIDDM's since they have lower levels of antioxidants and an increase in the more susceptible dense LDLs.

Two recent papers focused on vitamin E and endothelium-dependent relaxation. Keegan *et al.* (1995) used diabetic rats induced by streptozotocin for 2 months and found a 26.5% deficit in maximum endothelium-dependent vasorelaxation in diabetics. This deficit was reduced by 64.3% by vitamin E (1% of diet). They proposed that several key factors were involved: 1) vitamin E increases the prostacylin release which is decreased by high glucose concentration, 2) the increase in free radicals in diabetics has a direct effect on nitric oxide causing a reduction in vasorelaxation and 3) free radicals have a longer effect on endothelium integrity by causing the production of hydroxyl radicals that damage endothelium cells and increase the incidence of impaired neurovascular conduction.

Rosen *et al.* (1995) studied the hearts of streptozotocin induced rats. They also found the diabetic defect in endothelium-dependent vasodilation was prevented by pretreatment with vitamin E as well as a perfusion of the hearts with SOD. Vitamin E treatment was also seen to decrease the incidence of myofilament bundle destruction, numbers of focal necrosis areas, contraction bands and deposits of collagen fibers in the hearts. They concluded that

oxidative stress therefore has a role in endothelium dysfunction as well as destruction of cardiac structure and function in diabetics.

Another study on streptozotocin mice concentrated on peripheral nerve and neurovascular function (Cotter *et al.*,1995). Diabetes was shown to cause a decrease in sciatic motor conduction by 19.1% which was prevented 80-90% by vitamin E and beta carotene (both 1000mg/kg/day). Sciatic nutritive endoneurial blood flow decreased by 46.1% but was prevented 87%, 36% and 98% by vitamin E ,C and beta carotene respectively. It was speculated that free radicals are involved in decreasing the nutritive blood flow through peripheral nerve hypoxia and dysfunction. They found that a pharmaceutical dose was required in the amount of 100x greater than the rat diet. Their results were in contrast to another study (Nickander *et al.*, 1994) stating vitamin E had no effect, however the vitamin E concentration used in that study was less than 250mg/kg/day. The higher levels may be required in advanced diabetes because of associated advanced glycation and glucose autoxidation.

Vitamin C (ascorbic acid)

Young *et al.* (1995) focused on supplementation of streptozotocin treated rats (induced 1 week prior to the 6 week study) with ascorbate. They found that ascorbate alone did not reduce the oxidative stress but in combination with an iron chelator led to improvement in the malondialdehyde, conjugated dienes and antioxidant vitamins. One must be careful since ascorbate sometimes acts as a prooxidant, increasing oxidative stress. Ascorbate is known to increase iron absorption, and in combination with available transition metals can increase free radical production. Although ascorbate has been shown to inhibit glycosylation by competing with glucose on the protein (Davie *et al.*, 1992), ascorbate's oxidative product dehydroascorbate (DHAA) has been suggested to glycate proteins and compete with glucose for uptake into tissues. It has even been suggested that DHAA acts similarly to alloxan and destroys beta cells (Rose and Bode, 1993). In diabetics there is a depletion of ascorbate and an increase in dehydroascorbate (Young *et al.*,1992). Lower ascorbate levels may protect against lipid peroxidation by decreasing iron absorption, and therefore the metal catalysed production of the hydroxyl radical.

Micronutrients

Several studies have concentrated on the trace metals and micronutrients in relation to diabetes. Copper has been found to be increased in diabetics while zinc and magnesium are decreased (Walter *et al.*,1991). Copper is known to react with ascorbate to generate hydroxyl radicals (Rose and Bode, 1993). When magnesium was supplemented to streptozotocin (STZ)-diabetic rats (treatment occurring 1 week after STZ) no difference in glucose tolerance or myocardial function was seen compared to those diabetic rats not supplemented (Thompson *et al.*,1994). Thompson and Lee (1993) found streptozotocin-diabetic rats (4 week diet treatment following STZ induced diabetes) fed diets deficient in vitamin E and manganese had lower levels of antioxidant enzymes. The micronutrient Vanadium has been studied as a possible insulin-mimic in low doses. It appears to operate via a post-receptor mechanism (Thompson and Godin, 1995). When vanadyl sulfate was administered to STZ rats (12 week treatment after diabetic induction) a decrease in the development of cataracts (a complication of diabetes) was found (Thompson and McNeill, 1993). The role of trace metals and micronutrients in oxidative stress and diabetes is a relatively new area of study.

Plant Antioxidants

The accumulation of antioxidants in plants may be in defense to specific oxidative damage. There are numerous sources of oxygen metabolites within the plant. Photosynthesis in its production of oxygen and intermediate excited states is a prominent source. Respiration and nitrogen fixation also contribute to oxygen stress. Leghemoglobin, necessary for nitrogen fixation, in its oxygen reduced form is particularly susceptible to autoxidation. Stress factors such as heat, chilling, drought, water-logging, intense light, paraquat and certain air pollutants have been linked to increased production of antioxidant defenses (Dalton, 1995).

The plant's defenses to oxidative stress include carotenoids and tocopherols. The chloroplast has a high concentration of membranes and the tocopherols present in this organelle are especially important to prevent lipid peroxidation. Ascorbic acid and

glutathione are important in the ascorbate-glutathione pathway that scavenges hydrogen peroxide (Foyer *et al.*, 1994). Superoxide dismutases are present to convert superoxides to hydrogen peroxides while the catalase enzyme scavenges hydrogen peroxide. Reduction of superoxides and hydrogen peroxides prevents the formation of the damaging hydroxyl radical. Phytoferritin and phytic acid both decrease the availability of iron thereby preventing the catalysis of the production of hydroxyl radicals. Flavonoids, alkaloids and related secondary natural products have been studied in relation to decreasing lipid peroxidation.

There may be endogenous defenses in the chloroplasts of less light or temperature sensitive plants. Strong visible light and high temperature have been found to increase thylakoid lipid peroxidation in wheat especially when photoinhibition preceded temperature stress. This was determined to be a reaction to generated active oxygen species (Mishra *et al.*, 1993). Extrachloroplastic-accumulated secondary carotenoids were found to function in green alga by accumulating in lipid vacuoles around the nucleus thereby protecting the genome from free radical damage (Hagan *et al.*, 1993). In addition, the rice hulls from longer-lived rice have been found to have more phenolic hull constituents than other varieties. A C-glycosyl flavonoid with antioxidant properties similar to α-tocopherol was subsequently isolated from the hulls (Osawa *et al.*, 1992).

Considerable attention has been focused on plant products, especially those from traditional medicinal plants, that have antioxidant properties. The term antioxidant often refers to the ability to quench free radicals or singlet oxygen responsible for lipid oxidation. Lipid peroxidation, the damage caused by oxidants, frequently refers to the attack on cholesterol or phospholipids. Because these compounds are important to cell membrane structure this lipid oxygen toxicity has been linked to cellular malfunctions and subsequently an array of diseases.

Changes in the normal redox equilibrium of the oxidation-reduction reactions governing physiological processes can produce lipid peroxidation damage (Osawa *et al.*, 1992). The free radicals formed from organic radicals are mainly involved in the hydrophobic

membranous phase while those classified as oxygen-derived usually react in the extra- and intracellular fluids. The antioxidants can be either water-soluble (e.g.. ascorbic acid, uric acid, and thiols) or lipid-soluble (e.g.. vitamin E, flavonoids, and ubiquinols) (Tsuchiya *et al.*, 1992).

Natural antioxidants in plant foods can be endogenous, created during processing or as food additives. They can be free radical scavengers or quenchers of the formation of singlet oxygen as well as reducing agents and complexers of pro-oxidant metals. They are found as phenolics usually in the form of flavonoids (flavonols, isoflavones, flavones, catachins), cinnamic acid derivatives (including caffeic acid, ferulic acid and chlorogenic acid), coumarins, tocopherols, and polyfunctional organic acids (Pratt, 1992).

Many known antioxidants come from foods and beverages. Many medicinal plants are consumed as beverages and can therefore, as well, be considered a diet supplement. The minerals selenium, copper, zinc and manganese, and vitamins E (α-tocopherol), C and A (including carotenoids with provitamin A activity) are all nutrients which also exhibit antioxidant properties (Johns and Chapman, 1994).

Phenolic antioxidants have been found in numerous botanical extracts of foods, flavours, cosmetics and pharmaceuticals (Omar, 1992; Ho, 1992). Phenolic compounds are those with an aromatic ring and bearing one or more hydroxy substituents. When found in food these compounds are usually simple phenols, phenolic acids, hydroxycinnamic acid derivatives or flavonoids (Ho, 1992). Simple phenols and phenolic acids have been associated with certain fruits, cocoa beans, sesame oil, vanillin and tea. Hydroxycinnamic acid derivatives (commonly derived from p-coumaric, caffeic, ferulic or sinapic acid) have been studied in certain fruits, oilseeds, corn flour and Umbelliferous vegetables (Rommel *et al.*, 1992). Flavonoids (mainly catechins, proanthocyanins, anthocyanidins, flavones, flavonols and their glycosides) are found in numerous foods (Ho, 1992; Middleton, 1988).

The plant foods and spices studied for phenolic antioxidants include chili pepper, ginger, green tea, pepper, oregano, rice hull, rosemary, sesame seeds, soybean and thyme (Ho,

1992). Many are compared to tocopherols which have exhibited inhibition of lipid peroxidation *in vivo*, but have shown less promise as food antioxidants. The inhibition of lipoxygenase activity (arachidonic acid metabolism) has been associated with the inhibition of tumour promotion. Some phenolic antioxidants have exhibited this inhibition including a few of the oxidative dimers of tea catechins.

Numerous spices have been found to contain antioxidants in regard to food spoilage. Most studies have involved a comparison with α-tocopherol, or the synthetic antioxidants *tert*-butyl-4-hydroxyanisol (BHA) or *tert*-butyl-4-hydroxytoluene (BHT) in regard to the oxidation of lard or linoleic acid (Nakatani, 1992). The compounds, particularly phenolics, identified include phenolic diterpenes, phenolic carboxylic acids, biphenyls, flavonoids, phenolic amides, and diarylheptanoids. The antioxidant compounds known to occur in spices have some unusual properties: 1) the replacement of certain o-hydroxyl groups by methyl esters can inhibit the enzymatic browning that can be caused by some of these compounds, and 2) pH and temperature can determine their antithiamine activity (Pratt, 1992).

Antioxidant properties, as associated with food quality, have been found in the phenolic compounds of oilseeds. Research has been undertaken in regard to soybean and other oilseed flours (Shahidi, 1992). It has been reported that the inhibition of meat lipid oxidation by canola exceeds that of tocopherol and is comparable to BHT. Rapeseed/canola contains phenolic acids (including sinapic acid), tannins, flavonoids and lignins. The phenolic acids occur as hydroxylated derivatives of benzoic, cinnamic and coumaric acids and the tannins as polymers of flavan-3-ols or flavan-3,4-diols.

Oil-seed's antioxidant properties are associated with their flavonoid and hydroxylated cinnamic acids. The position and degree of hydroxylation of the ß ring of flavonoids as well as the ortho-dihydroxy grouping on one ring and the paradihydroxy grouping on the other (see structures of figure 4) has been found to be of major importance in their antioxidative ability (Pratt, 1992).

Assays and Models

Antioxidant Assays

There are numerous assays available to determine oxygen metabolite and antioxidant activity. The technology has developed along with instrumentation. Current studies on antioxidant activity now involve a series of experimental systems to determine the activity on different oxygen metabolites. A knowledge of the array of assays available is required to determine the specific antioxidant activity of any compound or plant.

An initial antioxidant assay arose from the desire to halt oxidation in food products. In 1951 Chipault *et al.* tested ground spices in lard. Solvent extracts of the spices were produced from the treatment of 25g of the spice fractionated with either the non-polar solvent, petroleum ether or the polar solvent, ethyl alcohol (95%). The stability of the sample was determined by the amount of time required to reach a peroxide value of 20 meq/kg. An antioxidant index was then determined by dividing the stability of the sample with the stability of lard without spice (6.5hours). A similar method using filter disks was used by Pratt and Watts (1963) and Pratt (1965) to study the antioxidant activity of vegetable extracts. Nakatani (1992) summarizes the natural antioxidants of certain key spices by their action of lowering the time required to oxidize linoleic acid rather than lard. The time required for the assay, however, was greater than 3 weeks.

Antioxidant indexes have also been produced from the ability to halt the bleaching of β carotene when in combination with a lipid emulsion. Taga *et al.* (1984) measured chia seed extracts in such a system at 15 minute intervals using a spectrophotometer. They also used agar diffusion plates to measure the bleaching time in hours around a hole containing the samples. The concentration of the phenolic antioxidants were then determined via thin layer chromatography (TLC), gas liquid chromatography (GLC) and ultraviolet spectral analyses.

There are many assays to evaluate lipid peroxidation. The most common is the TBARS or TBA (thiobarbituric acid) which involves thiobarbituric acid to measure malonaldehyde production by lipid peroxidation. Other methods include the use of HPLC (high

performance liquid chromatography), GLC or iodine's conversion to triodide to measure hydroperoxides. A decrease in NADPH absorbance can also be used to measure conjugated dienes (Girotti, 1990). Nourooz-Zadeh *et al.* (1995) devoted their work to developing a new method for measuring plasma lipid hydroperoxides. In their method ferrous oxidation with xylenol orange measured plasma lipid hydroperoxides in patients with NIDDM and found a higher concentration of lipid peroxides compared to control subjects. In parallel they found no difference in the indirect measurement of malondialdehyde. The method of choice appears to be one that measures the process directly.

An early antioxidant assay to measure lipid peroxidation utilized slices of beef covered with a test solution to measure malonaldehyde production (Pratt and Watts, 1963). Wilbur *et al.* (1949) describes this assay as using trichloroacetic acid and thiobarbituric acid solution to determine the difference in color production by various lipids and mouse skin. Frequently Ottolenghi (1959) is cited in relation to this procedure which is termed the TBA assay. Oxidation products from protein bound lipids and phospholipids are measured via heating with acid to produce malonaldehyde and its condensation with TBA (Tarladgis *et al.*,1960; Esterbauer and Cheeseman, 1990). Uchiyama and Mihara (1978) determined that the most reproducible TBA assay for animal tissues involves phosphoric acid with TBA and a double wavelength measurement to reduce interference. Free malonaldehyde has been measured via reaction with methylhydrazine and then analysis by GLC (Umano *et al.*, 1988).

The TBA assay has several significant limitations including the suppression by chelators, no detection of peroxidation of cholesterol and the production of TBA chromogens by compounds other than polyunsaturated fatty acids. These include ascorbic acid, certain sugars and certain amino acids (Girotti, 1985)- a definite negative therefore in studying plant antioxidants. Solubilization is also recommended of the samples to free membrane bound malonaldehyde and an antioxidant such as BHT should be added to reduce metal-catalyzed breakdown of lipid peroxides. The measurement of fluorescence as a detection of lipid oxidation has been found to be 10-100 times more sensitive than TBA (Logani and Davies, 1979).

The thiocyanate assay (TCA) is prominent in studies of Japanese origin. Osawa and Namiki (1981) used thiocyanate as a coloring agent to measure peroxidation in their study of *Eucalyptus* leaf fractions combined with linoleic acid. Results were measured over a 40 day period. Su *et al.* (1986) screened 195 crude plant drugs via methanol extracts of 0.2mg of plant powder. Twenty-two of these had stronger activity than α-tocopherol. Their analysis involved further fractionation and the use of thiocyanate yielding a +/- scale over >51 days. After Ramarathnam *et al.* (1989) studied rice hulls it was found that the thiocyanate results were better at separating activity from controls as compared to the TBA method (Osawa *et al.*,1992). Measurement occurred over 8-15 days. Jitoe *et al.* (1992) used the TCA method over 10 days to produce results and then proceeded to HPLC analysis to determine the curcuminoids present in tropical gingers. The time considerations in these cases seem prohibitory when doing a large number of assays.

HPLC is the method of choice to determine the exact amount of peroxidation products in regard to lipid peroxidation. Maruta *et al.* (1995) used HPLC analysis to measure methyl linoleate hydroperoxides produced from burdock root extractions mixed with methyl linoleate and an azo-initiator, 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN). Terao and Matsushita (1986) used HPLC to monitor the α-tocopherol effects on the autoxidation of methyl linoleate. Terao (1989) also used HPLC to study β-carotene's effect on the free radical oxidation (using AMVN) of methyl linoleate. Later Terao *et al.* (1992) published an article on assay procedures for antioxidant activity of carotenoids. These involved using HPLC to measure lipid hydroperoxides remaining after the carotenoid traps radicals. The radical initiators used were AMVN or 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) in methyl linoleate or phosphatidylcholine (PC) liposomes respectively. Terao *et al.* (1994) went on to use this approach to study specific flavonoid antioxidant activity in phospholipid bilayers.

Multilamellar and unilamellar liposomes, as artificial mediums, have been used to test photoperoxidation sensitizers as well as antioxidant activity (Terao *et al.*, 1992; Ratty *et al.*, 1988;Fleischer and Fleischer, 1989). Ratty *et al.* (1988) used liposomes in conjunction with a fluorescent probe to study the inhibition of non-enzymatic lipid peroxidation by

flavonoids. Liposomes were used because they were stable and did not have phase diagram shifts. A thin film of dipalmitoyl-DL-phosphatidylcholine (DPPC) was swollen with a Tris-base/sodium chloride buffer using glass beads while vortexing. This suspension then underwent ultrasonic irradiation in an oxygen-free nitrogen atmosphere. This procedure produced single, unilamellar vesicles with a diameter of about 700 angstroms. Terao *et al.* (1994) used unilamellar liposomes as a model system of biomembranes (phospholipid bilayers) to study the antioxidative effect of catechins. Preparation of the liposomes was similar to Ratty *et al.* (1988) with the following exceptions : 1) a solution of flavonoids was mixed with PC before creating the film under vacuum , 2) diethylene-triaminepentaacetic acid (DTPA) was added to prevent peroxidation by metal ions, and 3) after ultrasonic irradiation liposomes were created by passing the suspension through a membrane (the "extrusion method"). Peroxidation in these liposomes could then be measured via HPLC. Unilamellar vesicles have enabled a control over the lipid composition as well as the mode and extent of peroxidation for studies in the major detectable oxidation products of cholesterol (Sevanian and McLeod, 1987).

Animal *in vitro* methods to screen for antioxidants include the hemoglobin peroxidation test, liver microsomes, epidermal microsomes and liver mitochondria. Hemoglobin was used by Cort (1974) to speed up the lengthy tests in animal fats then used to measure antioxidant activity. Hemoglobin is used to peroxidize an oil which is monitored by an oxygen analyzer. The test is completed in minutes. Liver microsomes and mitochondria were used by Kagan *et al.* (1990) to study the hydrolysis of α-tocopherol derivatives. Rat liver mitochondria and microsomes, among other experimental systems, have been useful in the study of the antioxidant effects of tannins (Okuda *et al.*, 1992) and carotenoids (Palozza and Krinsky, 1992).

Ursini *et al.* (1994) used diphenyl-2-pycryl-hydrozyl radical (DPPH) as an initial screen of flavonoids for reactivity with a stable free radical. This method does not, however, rule out the possibility of producing free radical intermediates that also produce damage. They supplemented this screen with lipid peroxidation and carotenoid bleaching experiments. Okuda *et al.* (1992) used DPPH to study the scavenging activity of certain polyphenols in

addition to numerous lipid peroxidation assays. Ratty *et al.* (1988) also studied certain flavonoids using DPPH in addition to liposomal membrane and soybean lipoxygenase in order to determine the mechanisms of scavenging action.

The initial paper published regarding the use of DPPH was by Blois (1958). He stated that antioxidants had to be at a concentration of at least 10^{-5} molar to produce adequate results with this assay. The assay has been shown to work for cysteine, glutathione, ascorbic acid tocopherol, polyhydroxy aromatic compounds and aromatic amines. Glucose does not interfere but proteins tend to precipitate. Mellors and Tappel (1966) express some warnings regarding DPPH because they found it did not register butylated hydroxytoluene as a strong antioxidant. They suggest this was due to steric hinderance.

In addition to DPPH, Cotelle *et al.* (1996), used various experiments involving xanthine oxidase to study hydroxy-flavones. Chemiluminescence was used to monitor the amount of superoxide ions available in a xanthine oxidase system thereby determining the scavenging potential of the antioxidants. Several interesting papers (Kirby and Schmidt, 1997; Soares *et al.*1997) also used a superoxide scavenging assay in addition to that of free radicals. Superoxides are generated by a xanthine/xanthine oxidase system and monitored spectrophotometrically with nitroblue tetrazolium chloride (NBT). This is not to be confused with the study of the inhibition of xanthine oxidase which is used to produce uric acid. Studies on medicinal plants' inhibition of uric acid production have been used in the study of treatments for gout (Owen and Johns, 1999; Gonzalez *et al.*,1995).

Two different azo initiators, AAPH and AMVN, are often used to generate peroxyl radicals for the study of antioxidants. AAPH is considered hydrophilic and AMVN a lipophilic azo compound (Niki, 1990). They are often used to increase the oxidation of methyl linoleate in order to produce a system to study the reduction of oxygen uptake produced by antioxidants. Niki (1990) suggests that hydrophilic antioxidants only scavenge radicals in the aqueous phase while hydrophobic antioxidants only scavenge those oxidants produced within the membrane (ie. from AMVN).

Luminol has also been used to react with peroxyl radicals generated from AMVN in PC liposomes to produce a change in chemiluminescence (Marcoci *et al.*, 1994). Glazer (1990) used AAPH in conjunction with phycoerythrin fluorescence. The loss of fluorescence is an indication of free radical damage and antioxidant activity can be measured by slowing this loss. Tsuchiya *et al.* (1992) took this one step further by using a modified version of Glazer for an aqueous system and developing a hydrophobic system with AMVN, hexane and *cis*-parinaric acid as the fluorescent indicator. Another compound, 2,7-dichlorofluorescin-diacetate (DCF), is also used to monitor fluorescently the production of various oxygen radicals (Allan and Fluhr, 1997; Cathcart *et al.*, 1983). Using AAPH in a human serum system Valkonen and Kuusi (1997) determined DCF could also be used in a spectrophotometric assay.

The most current and thorough experiments on antioxidant activity involve numerous experimental systems. Cotelle *et al.*(1996) used different studies on xanthine oxidase and free radical scavenging (DPPH) as well as lipid peroxidation and autoxidation experiments. Marcocci *et al.* (1994) also used numerous systems in their study of ginkgo extracts and Tsuchiya *et al.* (1992) in their study of carotenoids and retinoids. In this way a more complete picture of antioxidant activity can be determined. In this thesis the antioxidant activity of the chosen plant's extracts were analyzed via three different assays: free radical scavenging using the DPPH system (Cotelle *et al.*,1996), superoxide scavenging using the NBT/xanthine oxidase system (Kirby and Schmidt, 1997) and peroxyl radical scavenging using a spectrophotometric assay containing AAPH and DCF (Vlakonen and Kuusi, 1997).

Antioxidants and Diabetic Models

There are various types of experimental models used to study the diabetic. The symptoms manifested by the model selected can have profound effects on the results. Most of the antioxidant studies in these models are directly related to the state of the pancreas, and therefore insulin production rather than resistance. Care must be exercised in choosing an appropriate model since conflicting results could be produced when compared to work using other models. The animal models most commonly used to study diabetes include the NOD (nonobese diabetic) mouse, BB (biobreeding/Winstar) rats and the MLDS (multiple-low-

dose streptozotocin) mouse or rat. The NOD mouse and BB rat, as well as the guinea pig and Chinese hamster, are all models of type 1 or insulin-dependent diabetes (IDDM). Models of NIDDM include the ob and db mice.

Both the ob and db mice have symptoms of obesity, hyperphagia, hyperglycemia and at least initial hyperinsulinemia. The ob mouse is said to be transiently hyperglycemic and not to have the pathologic changes such as damage to vascular, ocular and kidney systems associated with diabetes and oxidative stress. The db mouse, however, has these pathologies and hyperglycemic activity that culminates in death by ketosis at 10 months (Bell and Hye, 1983). There has been little to no current research on oxidative stress in diabetes using the db or ob mouse, usually human patients have been used in studying this area of NIDDM.

Antioxidant studies have been done using the MDLS model. The MDLS model involves chemically inducing diabetes in the animal. The two chemicals most commonly used on mice or rats to cause hyperglycemia are alloxan and streptozotocin. Both cause the destruction of beta cells (therefore a model of IDDM) and their action is mediated in some way by active oxygen radicals (Heineke *et al.*,1993). Alloxan (2,4,5,6-tetraoxohexahydropyrimidin) is the unstable form and is quickly reduced to the toxic dialuric acid. Streptozotocin (2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranose) or STZ is an antibiotic isolated from *Streptomyces achromogenes*. It is taken up by the islets of the pancreas and causes depression of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) (Oberley, 1988). MDLS uses STZ but contrary to a single dose of STZ it yields a model with initial lymphocytic insulinitis (Bell and Hye,1983). The glucose level of the animal given either of the diabetes inducing chemicals undergoes a triphasic response. Interpretation of results using these models should therefore not be done before 48 hours after administration of the chemical.

The inhibition of alloxan is involved in many studies, past and present. It is considered a model of free radical pathology. The action of alloxan is mediated by the hydroxyl radical produced by the Haber-Weiss reaction: $H_2O_2 + O_2^{\cdot-} = OH^{\cdot} + OH^{-} + O_2^{\cdot}$. Superoxide anion

radicals and hydrogen peroxide come from the reduction-oxidation cycle promoted by the reduced alloxan (from the unstable intermediate dialuric acid) (Grankvist *et al.*, 1981). The reduced alloxan is produced with superoxide anion which, when combined with iron, produces a Fenton type reaction producing hydrogen peroxide and hydroxyl radical as well as superoxide. The hydroxyl radicals likely play a role in the extensive DNA strand breakage and increased poly(ADP-ribose) synthetase found in alloxan toxicity (Halliwell, 1989).

Alloxan-induced diabetes is prevented by a number of direct or indirect reducing agents toward active oxygen species (Kojima *et al.*, 1992). Fisher and Hamburger (1980) emphasized that the diabetic action of alloxan is mediated by hydroxyl radicals generated from superoxide, hydrogen peroxide and iron. Pretreatment with superoxide dismutase, catalase, a chelator of iron, dimethyl sulphoxide, benzoate, mannitol, butanol, caffeine, theophylline, NADH or NADPH (the reduced forms of NAD and NADP respectively), and to a lesser extent, NAD^+ (the oxidized form of NAD), can protect against this action (Fisher and Hamburger, 1980; Grankvist *et al.*, 1979). In summary alloxan toxicity to the pancreatic beta cells is a product of the islets rapid uptake of alloxan as well as its sensitivity to active oxygen species (Oberley, 1988).

Alloxan-induced mice are also used to study antioxidant affects on diabetes. Stefek and Trnkova (1995) studied the effect of pretreating alloxan induced mice with pyridoindole stobadine and found it acted similar to all indole compounds by forming stable indolyl radicals as well as scavenging hydroxyl and peroxyl radicals. They state this chemical lowered the hypoglycemic action of alloxan. This means it halted the action of alloxan. Considering that they mention hydroxyl radicals as the responsible element for the action on alloxan, it followed that they focused on this chemical's similarity to the hydroxyl radical scavengers dimethyl sulfoxide, urea derivatives, butylated hydroxyanisole and amygdalin (as examined in other studies).

There have been many such experiments on testing new antioxidants on pretreated alloxan induced mice. In these cases the conclusions should emphasize the *in vivo* antioxidant

activity (possibly targeted to the pancreas) rather than any antidiabetic action. Heineke *et al.* (1993) studied another antioxidant in both NOD and MLDS injected mice. Their results include a decrease in prevalence in diabetes associated with the NOD mouse, however, their results varied depending on the age of the mouse, and an even greater decrease was seen when the antioxidant was combined with an immunosuppressant. In the other model (MDLS) the antioxidant was given prior to STZ and the results were as expected -a reduction of prevalence of diabetes. Heineke *et al.* (1993) conclude that the antioxidant prevented the onset of diabetes in two IDDM models. This is true but it does not necessarily mean it prevents diabetes.

Another recent study by Bellman *et al.* (1995) was on rat pancreatic islet cells *in vitro*. They found beta cell death can be caused by nitric oxide and reactive oxygen intermediates as mediators in cytokine production (specifically IL-1). They focused on the prediabetic inflammation state of the pancreas. IL-1 increases the oxidative stress in the islets as a consequence of the production of stress proteins. In addition, streptozotocin, nitric oxide and reactive oxygen species were found to increase DNA strand breaks. This causes the formation of poly(ADP-ribose) polymerase which further decreases the low state of NAD^+ in the islet cell. The depletion of NAD^+ causes an inhibition of proinsulin synthesis which is known to be a major cause of beta cell death (Heller *et al.*,1994; Halliwell, 1989; Oberley,1988). However, they found that the rapid production of a heat shock protein (hsp-70), unique to the islet cells, may compensate for those cell's lower radical scavenging potential (e.g. 10 fold lower glutathione) as well as lower amounts of NAD^+. The hsp-70 gene has been associated with IDDM.

It should be noted that before the destruction of beta cells there is an influx into the pancreas of mononuclear immuno-inflammatory cells. Since macrophage and lymphocyte inflammatory cells and their associated cytokines are toxic to beta cells, it has been suggested that a combination of antioxidant and immunosuppression therapy should be further championed (Heineke *et al.*, 1993). However, Kojima *et al.* (1992) found neopterin from monocytes and macrophages an effective scavenger of superoxide anion radicals and

active oxygen radicals. These immune factors could therefore be suppressing at least alloxan-induced mouse diabetes.

The research on antioxidants given to any of these models of diabetes provides little doubt that antioxidants that reach the pancreas can help prevent the damage caused to the susceptible beta cells from oxygen metabolites. Research into the destruction of the beta cells by oxygen metabolites, however, is only one piece of the puzzle in regard to oxidative stress and diabetes. There are other means to study antioxidant affects on diabetes, in particular NIDDM, where insulin resistance is a key factor.

The TNF in vitro *Model*

We chose to study the selected plants association to NIDDM through their interaction with tumor necrosis factor-α (TNF) production. In this manner we are looking at a possible affect on insulin resistance as well as diabetic complications. The concentration of TNF is elevated in the prediabetic as well as in those that already have NIDDM (Hussain *et al.*,1996). It is linked to insulin resistance, and therefore NIDDM, through its effect on the insulin-regulatable glucose transporter (Glut4) and the tyrosine kinase activity of the insulin receptor (Hotamisligil *et al.*, 1994). TNF can also increase reactive oxygen species already discussed as influential in the development of the complications of NIDDM. In addition TNF has been described as a key component in the obesity-diabetes link (Hotamisligil and Spiegelman, 1994).

TNF is circulated throughout the body in the bloodstream and produced by macrophage. It is a cytokine, a hormone-like protein, which is important in inflammation and cancer. Two of the major mechanisms involved in insulin use are affected by TNF. Glut4 is usually stimulated by insulin causing an increase in glucose transport into the cell. Glut4 mRNA levels, however, have been shown to be down regulated by TNF thereby decreasing insulin's effect (Hotamisligil and Spiegelman, 1994). In addition, studies involving the neutralization of TNF in an obese rodent model of diabetes found an increase in insulin stimulated phosphorylation of the insulin receptor. Since the insulin is bound to the receptor

through an autophosphorylation on multiple tyrosine residues, this led to the conclusion that TNF reduces the tyrosine kinase activity of the insulin receptor (Hotamisligil *et al.*,1994). In this way TNF affects diabetes by restricting the use of insulin by two different methods.

Considering that TNF is said to be a growth factor of adipocytes (fat cells) it has been thought to increase obesity (Hotamisligil and Spiegelman, 1994). TNF is also produced in excess by adipocytes. Since adipose tissue is often closely associated with skeletal muscle tissue and fibers it has been suggested that this tissue is providing the TNF to create insulin resistance in the muscle cells (Hotamisligil and Spiegelman, 1994). In addition, because TNF causes lipolysis and that the binding of TNF causes a decrease in free fatty acids (FFA), it is postulated that FFA may play an indirect role in TNF's affect on insulin resistance. FFA can stimulate hepatic glucose production as well as inhibit glucose uptake thereby contributing to hyperglycemia (Boden, 1997).

Antioxidants can decrease the detrimental affects of TNF on diabetes in two ways. Firstly they can scavenge the reactive oxygen species produced via TNF-induced mitochondrial production (Goossens *et al.*, 1995) and secondly by directly inhibiting TNF production and thereby decreasing insulin resistance. Some antioxidants are known to affect the production of TNF (Eugui *et al.*, 1994). In addition to Eugui *et al.*'s work on standard antioxidants, there has been a report on quercetin's activity (Sato *et al.*,1997) and numerous studies on the affects of vitamin C and E on TNF (Jeng *et al.*,1996, Bulger *et al.*,1997; Nakamura *et al.*,1998b).

Recently papers have been published using plant extracts or compounds derived from plants to suppress TNF production (Chang *et al.*,1995; Meloni *et al.*, 1995; Paul and Saxena, 1997). Most of these relate to plants used for rheumatism or immune problems. Considering the affect of antioxidants, as above, and the importance of TNF in diabetes we decided to test those selected plant extracts proven to be highest in antioxidant activity against TNF production.

Ethnobotany, Ethnopharmacology and Pharmacognosy

Ethnobotany refers to the study of the use of plants- including those used for medicine, foods, structures and crafts. The ethnobotanical sources used in this thesis to select the medicinal plants for study largely stemmed from the review of Arnason *et al.*(1981). This compilation of ethnobotanical studies concentrates on individual Indigenous Peoples of eastern Canada but also includes those of the Ojibwa and Cree. These studies include, Black (1980) on Algonquin ethnobotany, Smith (1932) on ethnobotany of Ojibwa, Chandler *et al.*(1979) on herbal remedies of the Maritimes, Strath (1903) on the pharmacy and therapeutics of the Cree and Densmore (1974) on the "Indian" use of wild plants for food, medicine and crafts. There are other sources of ethnobotany within Canada, many which also have information on the selected species, however this compilation served to identify 35 collectable plant species used for 3 or more of the selected symptoms.

Ethnobotany in Canada has been addressed by Turner (e.g., Turner, 1997) in numerous works largely related to food and beverages. She co-authored a book with Kuhnlein (Kuhnlein and Turner, 1991) on traditional plant foods that has provided some of the information on food and nutritional value of some of the selected medicinal plants in this thesis. The ethnobotany of the Chipewyan (Marles, 1984) likewise has additional information on the uses of many of the selected medicinal plants. Accounts of these plants were also found in Moerman's (1998) compilation of numerous works on the medicinal plants of North America with helpful cross-referencing to symptoms and cultures. Huthchins (1991), a smaller written account of herbology of North America, had helpful information in regard to some of the selected species. In addition the text by Erichsen-Brown (1979) was of great value in looking at the use of the selected plant species for the past 500 years.

Ethnobotany has enjoyed greater public interest with the growing concern of the continued destruction of rainforests and its affect on the world's plant biodiversity. The efforts of Schultes, Davis and Plotkin in their work in the Amazon largely on rubber and

hallucinogenics (Schultes, 1972; Schultes and Raffauf, 1990; Plotkin, 1993; Davis, 1996) has increased the profile of ethnobotany .

With the increased interest in alternative medicines and herbal medicines in particular, there has been an increased amount of research in ethnopharmacology (the cultural use of medicines) and pharmacognosy (the discovery of medicines or drugs from natural sources). Considering that one in four commercial drugs are derived from plant sources (Carlson *et al.*, 1997), commercial pharmaceutical companies are devoting more effort in these areas. Much of this work however uses high level through-put screening which screens a large collection of plants or specimens with a standard bioassay. Usually these plant collections do not make use of the knowledge of the local populations of the collection areas.

Research into the exact chemicals or compounds responsible for the effects and use of many Chinese medicinal plants can now be found in the *Journal of Ethnopharmacology* and the *Journal of Natural Products*. These studies make use of a long history of medicinal plant use as do those on plants used in India's ayurvedic medicines. Research in various parts of Africa have also studied the unique cultures and medicines relating to the local flora (e.g., Johns, 1994). Several databases, including the NAPRALERT system, now provide, for a fee, a compilation of much of the information on a specific plant provided by studies in ethnobotany and pharmacognosy.

This thesis draws on scientific works in the fields of ethnobotany, ethnopharmacology, pharmacognosy and general medicine. Information on the ethnobotany of the Indigenous Peoples of the boreal forest of Canada was gathered from the literature while the work done on the plants in the laboratory places this thesis in the realm of pharmacognosy. Most pharmacognosy works concentrate on the compounds or actions of one plant while this thesis studies 35 different plant species. The general conclusions based on the use of this group of plants therefore add to the knowledge of the use of plants for these symptoms in general and benefit the study of ethnopharmacology and ethnobotany.

Sociopolitical Considerations in Ethnopharmacognosy

Although the work involved in this thesis did not directly involve the gathering of information or plant material from Indigenous Peoples or communities, it is imperative to document the understanding of the importance of intellectual property rights in any work of this nature. Working with medicinal plants requires sensitivity to intellectual property rights. Sometimes getting ethnobotanical information from traditional herbalists is fraught with their fear of exploitation brought on by a history of abuses by Europeans. It is imperative to ensure the rights of those that give information essential for drug discovery. This is not only critical for ethical reasons but paves the way for collaborations in the future and decreases the chance of information being lost forever with the passing of a traditional healer.

Differences in culture can lead to misunderstandings of intentions as well as treatments afforded by the plants. When collecting material from developing countries differences in poverty levels and disease concerns (e.g.. diseases of affluence such as diabetes and heart disease) can lead to apathy towards the researcher from a developed country. In addition the development of pharmaceuticals out of the price range of the local communities of developing countries offers little incentive for collaboration (Elisabetsky, 1991).

Guidelines for collecting biological samples have recently been implemented through the Convention of Biological Diversity (CBD) as a product of the 1992 Rio Earth Summit. Conferences to evaluate case studies on benefit sharing arrangements has since been conducted by the United Nations (Moran,1998). Interpretation of the guidelines and their implementation can be as different as the partners involved. Some of the guidelines include respecting and preserving indigenous practices, obtaining prior informed consent, participation in research, providing scientific training, equitable sharing of benefits and encouraging the conservation of natural habitats and local biodiversity (Carlson *et al.*,1997).

Consent is required at the government, university or college, village and individual levels. If one wishes to import specimens, the government of the country must be fully informed and permits obtained in order to limit any consequences upon border crossings, which might include accusations of stealing (Elisabetsky, 1991). Consent and collaboration agreements are required from universities in the locality to facilitate the training and research participation conditions of the CBD. These collaborations can also lead to potential contacts with reputable herbalists or plant collectors (Cragg, 1994). Meetings with the governing bodies of the village should be undertaken prior to the research and collection processes (Scott and Receveur, 1995). This ensures the appropriate timing of the study thereby decreasing the disruption of local life (CBD's guideline of ensuring the respect and preservation of traditional lifestyle) and facilitates the discussion of compensation appropriate for the needs of the local population. Ideally government research institutions would match the research to an appropriate village interested in participating in such a study (Johns *et al.*, 1994). Obviously the final consent must come from the individual herbalist or healer. Agreements should cover the compensation expected both long term (i.e. royalties) and short term, the amount of publication, assurances of anonymity as required and the amount and form of data to be redirected to the participating individuals and institutions (Scott and Receveur, 1995; Cox,1990).

Short term compensation can take many forms depending on the circumstances. The individual healer may benefit with additions of food or collection tools in addition to monetary items to compensate for his/her time and effort. The village can be compensated with pharmaceuticals or hospitalization for those unsuccessfully treated by the herbalist. In combination with the training mandate compensation can also take the form of supplying teaching materials to village schools (Carlson *et al.*, 1997).

Long term compensation in the form of royalties means the return of benefits once a product is marketable. Shaman Pharmaceuticals, Inc. have approached this by developing a nonprofit trust fund, The Healing Forest Conservancy, to distribute any

royalties to all parties involved in their botanical pharmaceutical search. This fund will provide monies for projects that conserve the local biodiversity and traditional knowledge (Moran, 1998). Royalty payments are typically 5% of gross sales for basic materials and as high as 15% for identified product (Beese, 1997). In addition the academic involved in the instigation of the research shares any net royalties with his/her university at typical rate of 35:65 (McGill University:academic) or 50:50 (University of California) (Association of University Technology Managers,1995).

At the Centre of Indigenous Peoples' Nutrition and Environment (CINE) at McGill University work is conducted largely on traditional food systems following many of the guidelines of the CBD including informed consent, written agreements with community leaders, community training and confidentiality (Johns *et al*, 1994; Scott and Receveur, 1995). The work presented in this thesis did not involve the collection of information from a traditional community, elder or healer. The lab work was conducted on plants gathered locally and determined via literature search. Considering the information gathered through the literature is a filtered down version of traditional knowledge we are currently considering sharing any royalties that may arise from this project with CINE as a means of sharing the benefits with the original communities that gave the previously published information. Similar arrangements in the form of a fund have been suggested for those that use such databases of ethnobotany as NAPRALERT (Reid, 1996).

THE COLLECTION CRITERIA

The selection of species to collect was based solely on those used by the Indigenous Peoples living in the boreal forest of Canada for particular symptoms, specifically those used for three or more symptoms related to NIDDM. This protocol was followed in consideration that trading for particular plants growing outside the region of a particular group of people is not unknown, as well as some groups may live near the edge of the boreal forest and might collect species outside the boreal forest (Kuhnlein and Turner, 1991). Collection of species therefore was not limited to the boreal forest. The boreal forest of North America contains numerous groups of Indigenous Peoples that have used medicinal plants traditionally and, with the loss of their traditional lifestyle, have an increased incidence of diabetes. This area has a greater diversity of plant species (especially medicinal plant species) than that of the Arctic and a lower diversity and greater area than that of the rain forests of Canada's West Coast. In addition, the collection of species in or near the boreal forest would require minimal travel from the location of McGill University.

The Boreal Forest of Canada

The boreal forest of the world is a belt of woodland 1000km wide N-S running just south of the arctic tundra in North America, Europe and Asia. In Canada (figure 5) this area is the greatest vegetative region of the country with 299.2 million ha of forest land (Forestry Canada, 1989). The trees in this region are predominantly black and white spruce (*Picea mariana* and *glauca*, respectively) with lesser amounts of balsam poplar (southern ranges), larch, balsam fir, jack pine, hemlock, paper birch and trembling aspen. In the southern regions tall shrubs and herbaceous plants occur, with ferns, mosses and small winter green herbs under the thick forests. Bog and peat plants are also evident with a preponderance of lichens in the northern part of this range.

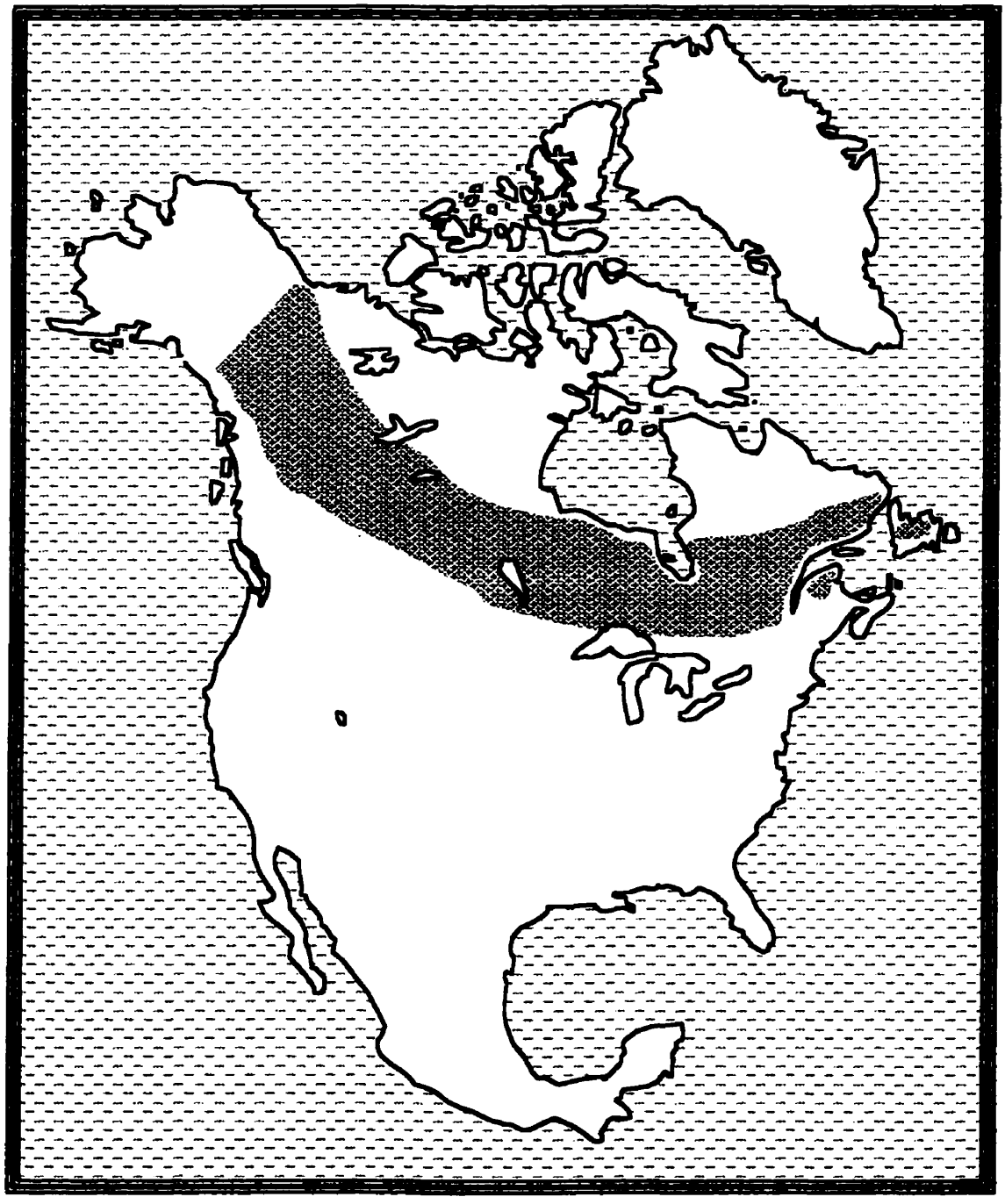


Figure 5. The boreal forest region of Canada

The climate in the boreal forest is considered humid microthermal with precipitation throughout the year in the form of rain and snow. Temperature means per month range from a low below minus 4°C to a high above 10°C. Mostly the area is considered subarctic with cool short summers of one to three months above 10°C (Department of Mines and Technical Surveys, 1957). Considering that the area was covered by glaciers until 12,000 years ago, the soils are relatively young. These are mostly podzol with some areas of grey or brown wooded soils and mountain soils with bits of subarctic soils in the Yukon and Alberta regions. There are rocky outcrops evident in parts of the boreal forest as well as areas of peat bogs (Department of Mines and Technical Surveys, 1957).

The Indigenous People

In the northwestern area of the boreal forest the groups of Indigenous Peoples include the Tutchone, Gwich'In, Kaska, Tagish, Dogrib and Slave. The Beaver, Chipewyan, Cree and Ojibway tend to live in areas further south and central while some Ojibway and Cree, as well as Algonquin and Montagnais, inhabit the eastern reaches of the boreal forest. Along the Atlantic coast there are some Peoples of the Malecite and Micmac. Most fall into two different major linguistic groups, the Athapaskan in the west and the Algonkian towards the east. Exceptions include the Montagnais (Iroquoian linguistic group) and the Tagish of the Tlingit group (Kuhnlein and Turner,1991; Department of Mines and Technical Surveys, 1957).

The traditional way of life for these people is as hunters and gatherers. Meat from the boreal forest included big game (e.g.. moose, caribou), smaller game such as beaver and rabbit, as well as fish, fowl and the occasional bear. Some of the Indigenous People were highly mobile in following the migratory patterns or range shifts of the caribou, salmon or moose (Helm,1981). A great variety of fruits, nuts and roots were gathered throughout the boreal forest region. Kuhnlein and Turner (1991) list over 500 plant species used within Canada for traditional food.

Following contact with Europeans the traditional way of life has deteriorated. The fur trade brought a reliance on guns and some market staples. The signing of treaties meant the loss of land and therefore hunting and gathering areas. The missionary schools continued to increase the need for market products by contributing to a taste for sugar, flour and lard at an early age (Kuhnlein and Receveur, 1996). The suppression of potlatchs and traditional medicinal practices further eroded the traditional lifestyle by decreasing a source of variety in foods or medicines (traded or given in potlachs) as well as a reliance on federally provided medicines. This decrease in the traditional lifestyle has led to a loss of food variety and a decrease in health (Kuhnlein and Receveur, 1996).

The Symptoms

A list of the 21 symptoms of diabetes and its complications used in the selection of plants are listed in Table 1 of manuscript C. Among these, heart disease, eye problems, kidney and peripheral neuropathies are the most destructive of these symptoms. Heart disease and angiopathy cause eighty percent of diabetic deaths. Diabetes is also the main cause of renal failure in the United States (27,900 new cases in 1995), the cause of most cases of blindness in the United States (diabetic cataracts) and responsible for 54,000 cases of amputations per year due to peripheral neuropathies (American Diabetes Association, 1998).

In addition to the oxidative stress factors in the complications of diabetes (discussed elsewhere), biochemical abnormalities account for differences in the blood vessels, nerves, kidneys and eyes of diabetics (Zeman and Hansen, 1991). Included in this are increased levels of glycoproteins and mucopolysaccharides. Glycoproteins are proteins that have an increased amount of attached carbohydrates and cause abnormal thickening of the basement membranes of the kidney and vascular system. Diabetics also have different mucopolysaccharides in their aorta, kidneys, skin and retinas.

The increased incidence of macroangiopathy in the diabetic account for the symptom selection of heart/chest pain, fatigue, circulation, blood purifier/blood tonic as well as in part, sores/wounds, abscesses/boils, inflammation and swelling. Macroangiopathy is

related to cardiovascular disease and the hardening of the arteries associated with atherosclerosis. This can reduce the circulation to the extremities making them more susceptible to infection and therefore amputations (Zeman and Hansen, 1991). Rheumatism/arthritis is included in this symptom grouping because of its similarity to some of the symptoms of macroangiopathy (e.g.. swelling of extremeties and fatigue and pain in said parts) thereby causing an underreporting of the above symptoms.

Microangiopathy refers to abnormalities of the capillaries where sclerosis can lead to thickening thereby decreasing circulation to such prone areas as the retina of the eyes, glomeruli of the kidneys and parts of the nervous system (Zeman and Hansen, 1991). Symptoms selected that relate to these aspects of the disease include back/kidney, urinary/diuretic, sore eyes and pain.

Neuropathies are common in diabetics and include such symptoms as pain, weakness, bladder problems (i.e. urinary/ diuretic symptom category), eye control, impotence (i.e. sexual irritability symptom category), abnormalities in the cardiovascular system and gastrointestinal upsets (including diarrhea) (Zeman and Hansen, 1991).

Pregnancy is listed since a difficult labor or pregnancy may signify gestational diabetes which often leads to larger babies. Symptoms related to urinary and renal problems (e.g.. infections) are commonly caused by the high levels of sugars in the urine of the diabetic. Weakness, fatigue (leading one to take a reviver/tonic) and headache are also common signs of diabetes related to abnormally fluctuating levels of glucose in the blood. Medicinal plants used for skin problems could be related not only to infection but the dehydration associated with diabetes. The category of general medicine/physic was included because of the numerous symptoms associated with diabetes as illustrated in the above descriptions.

INTRODUCTION TO MANUSCRIPT A

A list of thirty-five medicinal plants was created by selecting species used for 3 or more symptoms of diabetes or its complications by the Indigenous Peoples of the boreal forest of Canada. After gathering and drying these species, extracts were performed following a preliminary evaluation of extraction techniques (see appendix 1.). Considering oxidative stress is important in diabetes and its complications (Pisanti *et al.*, 1988; Paolisso *et al.*, 1993; Eriksson and Kohvakka, 1995; Gigliano *et al.*, 1995; Reaven, 1995; Salonen *et al.*, 1995), the following manuscript is a report on the antioxidant activities of the extracts of these chosen plant species. Three different antioxidant assays were used in manuscript A in order to get a more complete picture of total antioxidant activity within the extracts.

MANUSCRIPT A

Antioxidant activity in medicinal plants associated with the symptoms of diabetes mellitus used by the Indigenous Peoples of the North American boreal forest

Letitia M. McCune and Timothy Johns

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ABSTRACT

Thirty-five plant species were selected from the literature as used by the Indigenous Peoples of the boreal forest in Canada for 3 or more symptoms of diabetes or its complications. As oxidative stress is important in diabetes, 3 different antioxidant assays were performed on the plant extracts and compared to market produce, teas and coffee. Results of a DPPH assay of free radical scavenging activity found 89% had activity significantly greater than the market produce tested (Tukey, $P<0.05$), 14% were statistically equal to ascorbic acid and 23% had activities similar to green tea and Trolox®. Superoxides produced with an NBT/xanthine oxidase assay found scavenging was significantly higher in 29% of the species as compared to the market produce and a Trolox® positive control. Green tea performed in this assay similarly to *Rhus hirta*, *Quercus alba* and *Cornus stolonifera* (Tukey, $P<0.05$). Peroxyl radical scavenging was evaluated in a DCF/AAPH assay finding 60% of the species having activity statistically similar to Trolox® with *Rhus hirta* and *Solidago canadensis* having greater mean activities than green tea, ascorbic acid and Trolox®. The majority of the species (63% and 97%, respectively) had similar scavenging activities as compared to ascorbic acid in the superoxide and peroxyl radical scavenging assays. These results support the use of these traditional medicines in a lifestyle historically low in the incidence of diabetes.

Keywords: antioxidants, plant extracts, medicinal, DPPH, peroxyl, superoxide, diabetes

INTRODUCTION

Antioxidants are important in diabetes, with low levels of plasma antioxidants implicated as a risk factor for the development of the disease (Salonen *et al.*,1995; Pisanti *et al.*,1988) and circulating levels of radical scavengers impaired throughout the progression of diabetes (Collier *et al.*,1990; Godin *et al.*,1988). Many of the complications of diabetes, including retinopathy and angiopathy, the leading cause of mortality in diabetics, have been linked to oxidative stress (Baynes,1991) and recent literature considers antioxidants (i.e. vitamin E or C) as treatments (Giugliano *et al.*,1995; Paolisso *et al.*,1993; Salonen *et al.*,1995; Eriksson and Kohvakka,1995; Reaven,1995). Plants often contain substantial amounts of antioxidants, including vitamin E (tocopherols), carotenoids, ascorbic acid, flavonoids and tannins (Pratt, 1992; Larson, 1988) and we raise the possibility that antioxidant action may be an important property of plant medicines associated with diabetes.

The World Health Organization (Amos *et al.*, 1997) has estimated that by the year 2010 diabetes will affect 221 million people worldwide. Indigenous Peoples are 3-5 times more likely to develop the disease than the rest of the population (Canadian Diabetes Association, 1998; Young *et al.*, 1994), with most cases (90-95%) type 2 or non insulin dependant diabetes (NIDDM), a disease that is often controllable via diet and exercise. As an indigenous population changes from their traditional lifestyle their incidence of diabetes increases (Young *et al.*, 1994; Diamond, 1992; Brassard *et al.*, 1993) and in some groups (i.e. Pimas of Arizona) diabetes incidence is 50% or more for individuals between the ages of 30 and 65 (American Diabetes Association, 1997; Lillioja, 1996).

Plants are often the most common form of medicine for many indigenous people throughout the world and the hypoglycemic action of plants used by various indigenous people for diabetes has been reviewed (Ernst 1997; Marles and Farnsworth, 1995; Bailey and Day, 1989; Perl, 1988). Considering that 81% of 295 plants traditionally used for diabetes–related conditions have the ability to lower blood glucose (Marles and Farnsworth, 1995), there is a degree of scientific validity to traditional practices. To the

best of our knowledge plants in Canada have not been scrutinized in relation to use for diabetes or hypoglycemic activity.

Past traditional lifestyles, in which diet, exercise and possibly antioxidant and hypoglycemic medicines played an important role, may have masked people in a pre-diabetic state. As an evaluation of the benefit of the traditional lifestyle in relation to diabetes we chose to study three types of antioxidant activity in those plants used by the Indigenous Peoples of the North American boreal forest for any symptoms associated with diabetes or its complications. On the premise that the greater the number of symptoms a particular species is used for, the greater its relevance to diabetes (Carlson, personal communication), we looked at all species used for 3 or more symptoms of diabetes or its complications. Considering that the loss of the use of native plants for food or medicine is accompanied by an increase in consumption of market products (Kuhnlein and Receveur, 1996), some commonly consumed market produce (carrots, lettuce and potato) and beverages (teas, coffee and wine) were included in this analysis for comparative purposes.

Considering that the use of medicinal plants were usually in the form of tea all of our assays were related to water soluble components. We assessed antioxidant activity initially as free radical scavenging activity. The 1,1 diphenyl-2-picryl-hydroazyl (DPPH) assay used for hydrogen donating capacity is commonly employed for screening plant extracts (Kirby and Schmidt, 1997; Soares *et al.*,1997; Kang *et al.*,1997; Mathiesen *et al.*,1995). This assay, however, does not discount possible reactions with free radical intermediates and the production of further oxidative chain reactions. A second assay measured scavenging activity against peroxyl radicals, components of the chain reactions commonly associated with lipid peroxidation, hydrogen peroxide production and atherosclerosis (Dix and Aikens, 1993; Halliwell, 1997). An assay using 2,7-dichlorofluorescin-diacetate (DCF) measured scavenging activity rather than TBARS considering inhibitors of the TBARS assay (e.g. ascorbic acid) may have been present in the crude plant extracts (Girotti, 1985) and a more general and direct assay was desired (Logani and Davies, 1979). A third assay assessed superoxide scavenging activity.

Superoxides are important in the ischemic tissue injury commonly associated with diabetic microangiopathy (McCord,1985; Thompson and Godin,1995) and as potential initiators of the chain reactions that can produce hydrogen peroxide.

METHODS

Reagents

Trolox®, the flavonoid standards, hypoxanthine and DPPH were purchased from Sigma/Aldrich (St. Louis, MO), L-ascorbic acid and EDTA from BDH (St. Laurent, Que.), Chelex® resin from Bio-Rad (Hercules, CA) and AAPH from Polyscience (Niles, IL). Nitroblue tetrazolium chloride and 2,7 dichlorofluorescein-diacetate (DCF) were obtained from Fisher/Acros (Montreal, Que.).

Species collection

Plant species to be screened were identified according to a literature review concentrating on those species used by the Indigenous Peoples of the North American boreal forest in relation to a total of 21 symptom categories (McCune and Johns, manuscript C). Only species used for three or more symptoms of diabetes or its complications were selected. Collection sites were not necessarily located in the boreal forest but represent medicinal species used by people living within the boreal forest. Medicinal species selected but known to grow in more southern ranges (e.g., *Sassafras albidum)* were collected to analyze with the speculation that these species may have been acquired via trade with people living further south (Kuhnlein and Turner, 1991). All plant specimens were collected in the months of June through September of 1996 and 1997. Samples represent a collection of plants (≤ one kg of dried material) from a single population. Specific site locations are indicated in Table 1. Only the plant part (bark, flowers, fruit, leaves or root) most used for the selected symptoms was analyzed for each species. Voucher specimens are deposited in the McGill University Herbarium (Ste. Anne de Bellevue, Canada).

Market produce (carrots, potatoes, iceberg lettuce and garlic) was purchased locally. Green tea was of the brand Twinings, black tea was from Salada, coffee was Maxwell House-extra

Table 1. Plant species collected in relation to the treatment of the symptoms of diabetes and its complications

Species used medicinally (common name), voucher ID	locality*	plant part	extract yield w/w (%)	Indigenous Peoples**
Abies balsamea (balsam fir), 18	St. Eugène, Ontario	bark/twigs	13.2	Ojibwa, Algonquin, Montagnais, Micmac
Achillea millefolium (yarrow), 15	Hudson, Quebec	flowers	24.4	Ojibwa, Algonquin, Cree
Acorus calamus (sweet flag), 24	Vaudreuil, Quebec	roots	14.1	Iroquois, Ojibwa, Algonquin, Malecite, Cree
Aralia nudicaulis (wild sarsaparilla), 7	Ile Perrot, Quebec	roots	9.1	Ojibwa, Algonquin, Abenaki, Montagnais
Aralia racemosa (spikenard), 19	Ile Perrot, Quebec	roots	18.8	Ojibwa, Micmac, Malecite
Arisaema triphyllum (indian turnip), 3	Vaudreuil, Quebec	roots	5.8	Ojibwa, Malecite
Asarum canadense (wild ginger), 12	Ile Perrot, Quebec	roots	17.2	Ojibwa, Montagnais
Celastrus scandens (climbing bitter-sweet), 8	Baie d'Urfé, Quebec	roots	4.4	Iroquois, Ojibwa
Cornus stolonifera (red-osier dogwood), 9	Ste. Anne de Bellevue, Quebec	bark	20.0	Ojibwa, Algonquin, Abenaki, Malecite
Corylus cornuta (beaked hazelnut), 28	Sawyerville, Quebec	bark/twigs	12.0	Algonquin, Abenaki, Cree

Table 1. ***(continued)***

Species used medicinally (common name), voucher ID	locality	plant part	extract yield w/w (%)	Indigenous Peoples**
Dirca palustris (moosewood), 20	Hudson, Quebec	root bark	15.3	Iroquois, Ojibwa
Gaultheria procumbens (wintergreen), 13	Ile Perrot, Quebec	leaves	37.6	Iroquois, Ojibwa, Algonquin, Cree
Heracleum lanatum (cow parsnip), 23	Sawyerville, Quebec	roots	21.4	Ojibwa, Micmac, Cree
Juniperus communis (juniper), 30	Arnprior, Ontario	bark	19.7	Micmac, Malecite, Cree, Delaware
Juniperus virginiana (red cedar), 31	Bay of Quinte, Ontario	leaves/twigs	34.4	Ojibwa, Cree
Kalmia angustifolia (sheep laurel), 11	Ile Perrot, Quebec	leaves/flowers	47.4	Montagnais, Micmac, Malecite, Cree
Ledum groenlandicum (Labrador tea), P. Owen#15	Waskaganish, Quebec	leaves	23.2	Ojibwa, Algonquin, Montagnais, Micmac, Malecite, Cree
Nuphar variegatum (yellow water-lily), 32	Hudson, Quebec	roots	32.0	Iroquois, Algonquin, Abenaki, Micmac
Picea glauca (white spruce), 17	St.. Eugène, Ontario	bark/twigs	8.9	Ojibwa, Algonquin, Montagnais, Abenaki, Micmac, Cree
Picea mariana (black spruce), 16	Hudson, Quebec	leaves	31.5	Ojibwa, Algonquin, Cree

Table 1. ***(continued)***

Species used medicinally (common name), voucher ID	locality	plant part	extract yield w/w (%)	Indigenous Peoples**
Populus balsamifera (balsam poplar), 27	Sawyerville, Quebec	buds	36.7	Ojibwa, Algonquin, Micmac, Malecite, Cree
Populus tremuloides (quaking aspen), 14	Ste. Anne de Bellevue, Quebec	bark	11.6	Ojibwa, Cree, Abitibi
Prunus serotina (rum cherry), 22	Sawyerville, Quebec	bark	25.5	Iroquois, Ojibwa, Malecite, Delaware
Quercus alba (white oak), 33	Woburn, MA, USA	bark	17.3	Ojibwa, Micmac, Malecite, Delaware
Quercus rubra (red oak), 6	Ile Perrot, Quebec	bark	9.9	Ojibwa, Malecite
Rhus hirta (staghorn sumac), 4	Ile Perrot, Quebec	fruit	36.8	Iroquois, Algonquin, Malecite, Delaware
Sassafras albidum (sassafras), store purchase		bark	4.1	Iroquois, Ojibwa
Smilacina racemosa (false spikenard), 2	Vaudreuil, Quebec	roots	32.2	Ojibwa, Abenaki, Algonquin
Solidago canadensis (goldenrod), 29	Ile Perrot, Quebec	roots	19.4	Ojibwa
Sorbus americana (mountain ash), 25	Batchewana Bay, Ontario	bark	17.2	Ojibwa, Algonquin, Montagnais, Malecite, Cree

Table 1. ***(continued)***

Species used medicinally (common name), voucher ID	locality	plant part	extract yield w/w (%)	Indigenous Peoples**
Taraxacum officinale (dandelion), 1	Ste. Anne de Bellevue, Quebec	roots	15.1	Iroquois, Ojibwa, Algonquin, Cree
Taxus canadensis (yew), 26	Batchewana Bay, Ontario	twigs/leaves	36.8	Ojibwa, Algonquin, Montagnais, Abenaki, Micmac, Malecite
Thuja occidentalis (arbor vitae/white cedar), 10	Phillipsburg, Quebec	leaves	22.6	Iroquois, Ojibwa, Algonquin, Montagnais, Abenaki, Malecite, Micmac
Tsuga canadensis (hemlock), 5	Ile Perrot, Quebec	bark	15.7	Ojibwa, Algonquin, Abenaki, Malecite, Micmac
Verbascum thapsus (mullein), 21	Mt. Rigaud, Quebec	roots	15.7	Iroquois, Ojibwa, Malecite

*Amprior, Ontario 45° 26′ N, 76° 21′ W; Baie D'Urfe 45°25′ N, 73°55′ W; Bay of Quinte, Ontario 44° 09′ N, 77° 15′ W; Batchewana Bay, Ontario 46° 56′ N, 84° 36′ W; Hudson, Quebec 45° 30′ N, 74° 09′ W; Ile Perrot, Quebec 45° 23′ N, 73° 57′ W; Montreal, Quebec 45° 24′ N, 73° 36′ W; Mt. Rigaud, Quebec 45° 27′ N, 74° 18′ W; Phillipsburg, Quebec 45° 02′ N, 73° 05′ W; Sawyerville, Quebec 45° 21′ N, 71°28′ W; St. Eugene, Ontario 45° 30′ N, 74° 28′ W; Ste. Anne de Bellevue, Quebec 45° 25′N, 73° 56′ W; Vaudreil, Quebec 45° 24′ N, 74° 02′ W; Waskaganish Quebec 51° 37′ N, 78° 50′ W; Woburn, MA, USA 42° 29′ N, 71° 09′ W.

** Listed are only those Indigenous Peoples within the boreal forest region of Canada that used the species for 3 or more diabetes related symptoms as listed in Arnason *et al.*,1981

fine grind-100% pure coffee and the red wine was Notre Vin Maison from Les Vins La Salle.

Extract Preparation

Samples were air-dried in the shade before grinding in a Wiley mill to a particle size of 850μm. Some of the thicker root samples and the market produce were frozen and dried via lyophilization before the grinding procedure. Extractions were performed in methanol (HPLC grade) using a Soxtec extractor at the rate of 10g of powdered sample to 210ml methanol over a period of 3 hours. Methanol and moisture were removed via rotoevaporation and lyophilization. The red wine sample was not extracted, the 350ml of wine had the alcohol and water removed via rotoevaporation and lyophilization. Extract yields were determined from the original weight of ground sample before extraction over the weight of extract following rotoevaporation and lyophilization. The concentrated extracts were stored dry at -20^0C in amber jars with teflon-lined caps.

DPPH Assay

This method is similar to that of Cotelle *et al.* (1996) with the substitution of methanol instead of ethanol. A solution of 3.0 ml 100 μM DPPH solution in methanol was gently mixed with 0.5 ml of plant extract (at various concentrations) in methanol. After 10 minutes the absorbance at 517 nm was compared to a control of methanol. The IC_{50} (the concentration required to inhibit or change the absorbance by 50%) was calculated as an absorbance change of 0.4. This 0.4 change was determined from a standard IC_{50} generated from the scavenging action of ascorbic acid at 29 μM (Ursini *et al.*,1994).

NBT/XO (superoxide scavenging)Assay

The scavenging potential for superoxide radicals was analyzed via a hypoxanthine/xanthine oxidase (XO) generating system coupled with nitroblue tetrazolium (NBT) reduction (measured spectrophotometrically) following the method of Kirby and Schmidt (1997). The reaction mixture contained 125 μl buffer (50 mM KH_2PO_4/KOH, pH 7.4), 20 μl of 15 mM Na_2 EDTA in buffer, 30 μl of 3 mM hypoxanthine in buffer, 50 μl of 0.6 mM NBT in buffer, 50 μl of xanthine oxidase in

buffer (1 unit per 10 ml buffer) and 25 µl of plant extract in buffer (a diluted sonicated solution of 10 µg per 250 µl buffer). Microplates of 96 wells were read 2.5 minutes after the addition of xanthine oxidase at 540 nm. Superoxide scavenging activity was expressed as % inhibition compared to the blank (buffer in place of extract).

DCF/AAPH Assay

A water soluble azo initiator [2,2'-diazobis(2-amidinopropane)dihydrochloride or AAPH] produced the peroxyl radicals while a spectophotometric analysis of 2,7-dichlorofluorescin-diacetate (DCF) monitored the scavenging activity of the plant extracts (Vlakonen and Kuusi, 1997). The activation of DCF followed that of Cathcart *et al.*(1983) where 350 µl of a 1 mM stock of DCF in ethanol was mixed with 1.75 ml of 0.01 N NaOH and allowed to stand for 20 minutes before the addition of 17.9 ml of 25 mM sodium phosphate buffer (pH 7.2). Chelex 100 Resin was used to remove any contaminating metals from the buffer. The reaction mixture contained 25 µl of plant extract in methanol (to a final concentration of 2 µg/ml), 150 µl of activated DCF solution and 25 µl of AAPH (to a final concentration of 56 mM). The reaction was initiated with the addition of the AAPH. Absorbance was read at room temperature every 4 minutes at 490nm with the subtraction of background at 590 nm. Results were calculated over 60 minutes from the area under the curve and % inhibition was determined from the comparison to the buffer control curve recorded with each reading.

Statistics

Tukey analysis was conducted on the means of antioxidant activity using SAS® software. Analysis of the DPPH results was achieved using a transformation of log(y+1). Differences were determined using a $P<0.05$. In all cases antioxidant activity was based on at least three independent experiments performed in duplicate.

RESULTS

DPPH free radical scavenging

Eighty-nine percent of the plant species extracts tested (Table 2) had significantly greater activity (lower IC_{50}, $P<0.05$) in the DPPH assay than the market produce tested and shown in Table 3. Three of these had mean activities greater than, but statistically equal to, green tea. *Rhus hirta* was the only species with a mean activity greater than vitamin C (ascorbic acid). Of the species tested, 14% had activity statistically similar to ascorbic acid and 23% similar to green tea and trolox® (a water soluble analogue of α-tocopherol or vitamin E). Coffee showed some activity in this assay and black tea fell between coffee and green tea in the beverages. Like the market produce the red wine sample had little or no activity. The standard polyphenolics (quercetin, epicatechin and tannic acid in Table 3) all performed well in this assay, but not as well as *Rhus hirta.*

NBT/XO superoxide scavenging

As shown in Table 2 and 3, the majority, 63% of the medicinal species, had superoxide scavenging activities similar to ascorbic acid with nine of the species having mean activities greater than the ascorbic acid positive control. Only one of these (*Rhus hirta*) was significantly greater. Twenty-nine percent of the species tested had activities significantly higher (Tukey analysis, $P<0.05$) than the market produce and a trolox® positive control. Trolox® reacted similarly to the produce with little to no activity. Green tea performed similarly to *Rhus hirta*, *Quercus alba* and *Cornus stolonifera* (Tukey analysis, $P<0.05$).

DCF/AAPH peroxyl radical scavenging

Two of the species, *Rhus hirta* and *Solidago canadensis* had higher mean peroxyl radical scavenging activity than green tea, ascorbic acid and trolox®. Their activity (% inhibition) was significantly greater ($P<0.05$) than 43% of the plant species extracts and the market produce tested. Sixty percent of the species had activity statistically similar to trolox®. The market produce (Table 3) had next to no activity in this assay. Green tea,

Table 2. Antioxidant activity of 35 plant species traditionally used for symptoms of diabetes or its complications

	DPPH Assay (IC_{50} in ppm)*		NBT/XO Assay (%inhibition)		DCF/AAPH Assay (%inhibition)	
Crude Extract	Mean +/- S.E.		Mean +/- S.E.		Mean +/- S.E.	
***Rhus hirta* fruit**	3.73 +/- 0.07	a**	44.49 +/- 1.23	ab	31.49 +/- 2.81	a
***Cornus stolonifera* bark**	5.72 +/- 1.03	ab	34.53 +/- 1.22	abcd	18.32 +/- 0.65	abcdefg
***Nuphar variegatum* roots**	6.02 +/- 0.59	abc	24.30 +/- 1.72	cdefg	18.91 +/- 0.49	abcdef
***Quercus alba* bark**	8.17 +/- 0.74	bcde	35.76 +/- 0.45	abc	22.41 +/- 2.96	abc
***Corylus cornuta* bark/twigs**	8.82 +/- 0.23	bcdef	19.64 +/- 2.24	efghijkl	20.96 +/- 1.08	abcde
***Kalmia angustifolia* leaves/flowers**	11.44 +/- 2.60	cdefg	22.83 +/- 2.52	cdefgh	20.04 +/- 3.23	abcdef
***Quercus rubra* bark**	11.55 +/- 1.08	defg	25.24 +/- 0.60	cdef	18.05 +/- 2.61	abcdefg
***Juniperus communis* bark**	11.82 +/- 2.33	defg	31.45 +/- 1.86	bcde	21.93 +/- 2.04	abc
***Picea mariana* leaves**	13.57 +/- 0.76	efgh	15.11 +/- 2.68	fghijklmno	15.62 +/- 3.96	abcdefghi
***Sorbus americana* bark**	15.80 +/- 1.91	fgh	18.41 +/- 1.76	efghijklm	16.75 +/- 4.03	abcdefghi
***Gaultheria procumbens* leaves**	16.39 +/- 2.79	fgh	10.04 +/- 2.86	ghijklmnop	9.79 +/- 3.45	cdefghi
***Tsuga canadensis* bark**	17.01 +/- 1.71	ghi	24.69 +/- 2.50	cdef	18.83 +/- 1.52	abcdefg
***Prunus serotina* bark**	17.78 +/- 3.92	ghi	20.86 +/- 0.68	defghijk	19.70 +/- 0.60	abcdef
***Picea glauca* bark and twigs**	19.55 +/- 0.89	ghij	15.74 +/- 1.60	fghijklmn	21.49 +/- 2.74	abcd
***Ledum groenlandicum* leaves**	21.92 +/- 1.82	hij	12.76 +/- 2.19	fghijklmnop	21.33 +/- 0.76	abcd
***Abies balsamea* bark/twigs**	22.16 +/- 1.05	hij	22.48 +/- 0.43	cdefghi	17.53 +/- 1.26	abcdefgh

Table 2. *(continued)*

Crude Extract	DPPH Assay (IC_{50} in ppm)* Mean +/- S.E.		NBT/XO Assay (%inhibition) Mean +/- S.E.		DCF/AAPH Assay (%inhibition) Mean +/- S.E.	
***Sassafras albidum* bark**	23.92 +/- 0.64	hijk	8.37 +/- 1.71	ijklmnop	16.23 +/- 3.83	abcdefghi
***Juniperus virginiana* leaves/twigs**	29.76 +/- 2.47	ijkl	9.08 +/- 1.93	hijklmnop	16.09 +/- 1.09	abcdefghi
***Thuja occidentalis* leaves**	31.60 +/- 2.17	jkl	17.27 +/- 2.52	efghijklm	10.83 +/- 2.63	cdefghi
***Solidago canadensis* roots**	42.51 +/- 3.83	klm	18.43 +/- 1.81	efghijklm	31.32 +/- 2.89	a
***Populus balsamea* buds**	42.81 +/- 4.64	klm	8.02 +/- 2.24	jklmnop	11.63 +/- 0.63	cdefghi
***Taxus canadensis* twigs/leaves**	51.09 +/- 3.63	lmn	9.75 +/- 2.77	hijklmnop	9.35 +/- 5.49	cdefghi
***Achillea millifolium* roots**	67.64 +/- 2.75	mno	7.13 +/- 0.74	klmnop	16.61 +/- 1.74	abcdefghi
***Verbascum thapsis* roots**	67.80 +/- 1.95	mno	8.07 +/- 1.63	jklmnop	10.31 +/- 3.98	cdefghi
***Acorus calamus* root**	75.71 +/- 5.89	no	1.34 +/- 2.44	op	19.17 +/- 2.57	abcdef
***Aralia nudicaulis* roots**	79.04 +/- 1.97	no	-0.37 +/- 3.48	p	10.88 +/- 3.96	cdefghi
***Asarum canadensis* roots**	87.78 +/- 2.82	nop	6.94 +/- 1.31	klmnop	11.88 +/- 1.94	cdefghi
***Celastris scandens* roots**	91.33 +/- 3.84	op	1.70 +/- 1.85	nop	11.28 +/- 4.61	cdefghi
***Populus tremuloides* stem bark**	102.90 +/- 4.06	op	7.25 +/- 0.79	klmnop	12.76 +/- 1.99	bcdefghi
***Aralia racemosa* roots**	152.10 +/- 5.44	pq	0.08 +/- 2.29	p	7.95 +/- 4.21	cdefghi
***Dirca palustris* root bark**	233.28 +/- 7.94	qr	-0.30 +/- 1.75	p	6.65 +/- 1.44	cdefghi
***Arisaema triphyllum* corms**	291.94 +/- 9.63	rs	1.00 +/- 1.73	op	8.44 +/- 3.07	cdefghi
***Taraxacum officinalis* roots**	347.59 +/- 7.80	rs	6.26 +/- 2.82	lmnop	12.06 +/- 2.93	cdefghi

Table 2. *(continued)*

	DPPH Assay (IC_{50} in ppm)*		NBT/XO Assay (%inhibition)		DCF/AAPH Assay (%inhibition)	
Crude Extract	Mean +/- S.E.		Mean +/- S.E.		Mean +/- S.E.	
***Smilacea racemosa* roots**	624.36 +/- 58.82	tu	-1.04 +/- 2.76	p	1.77 +/- 3.01	hi
***Heracleum lanatum* root**	626.03 +/- 36.67	tu	-0.27 +/- 1.97	p	7.74 +/- 4.09	cdefghi

*the inhibitory concentration that caused the same 50% decrease as ascorbic acid

**means with the same letter within an assay are not significantly different as determined by Tukey analysis($P<0.05$)

Table 3. Antioxidant activity of common beverages, vegetables and standards

	DPPH Assay (IC_{50} in ppm)		NBT/XO Assay (%inhibition)		DCF/AAPH Assay (%inhibition)	
Crude extract	Mean +/- S.E.		Mean +/- S.E.		Mean +/- S.E.	
Green tea	6.76 +/- 0.14	abcd	46.35 +/- 0.93	a	28.59 +/- 2.03	ab
Black tea	15.19 +/- 0.97	fgh	26.27 +/- 5.28	cdef	11.30 +/- 0.91	cdefghi
Coffee	40.32 +/- 5.12	klm	17.63 +/- 3.87	efghijklm	13.66 +/- 3.47	bcdefghi
Red wine	240.05 +/- 34.70	qr	9.39 +/- 2.63	hijklmnop	5.23 +/- 2.04	efghi
Carrots	831.51 +/- 163.94	tu	5.87 +/- 3.09	lmnop	6.63 +/- 2.91	cdefghi
Potatoes	521.57 +/- 1.37	st	6.28 +/- 2.10	lmnop	4.57 +/- 3.94	fghi
Iceburg lettuce	1064.34 +/- 167.59	u	6.18 +/- 2.45	lmnop	2.96 +/- 1.55	ghi
Garlic	833.28 +/- 96.97	tu	5.20 +/- 2.72	mnop	1.06 +/- 0.93	i
Ascorbic acid	5.16 +/- 0.18	ab	22.29 +/- 1.14	cdefghij	20.32 +/- 1.79	abcde
Trolox*	8.97 +/- 2.11	abcd	5.49 +/- 0.91	lmnop	24.16 +/- 1.52	ab
Quercitin	6.09 +/- 0.09		ND		ND	
Epicatechin	6.34 +/- 0.00		ND		ND	
Tannic acid	5.81 +/- 0.04		ND		ND	

*alpha-tocopherol was used in the DPPH assay rather than Trolox

ND=not determined

ascorbic acid and trolox® all reacted comparatively well against peroxyl radicals. On the basis of the other assays *Solidago canadensis*'s high activity was unexpected. Analyzing the ranks of the three assays using the Kruskal-Wallis method significant differences were found between the rankings. In particular, the results for *Gaultheria procumbens* and *Solidago canadensis* in the DCF assay causes a considerable shift of these species in an overall ranking –*Gaultheria procumbens* to position 19 of 35 (compared to 11 in the DPPH assay) and *Solidago canadensis* to position 11 (as compared to 20 in the DPPH assay).

DISCUSSION

The selection of plants based on diabetes symptoms identified numerous species with high antioxidant potential with 89% of these traditional plant medicines having greater overall free radical antioxidant activity than the basic market produce tested. As well, 29% of the species had activity against superoxides greater than the market produce and many did better than the produce against peroxyl radicals.

Rhus hirta was the best scavenger in all three assays, while *Cornus stolonifera, Nuphar varigatum, Quercus alba* and *Solidago canadensis* were also excellent scavengers in one or more assays. Phenolics, in particular flavonoids, are often directly linked to antioxidant activity (Cao *et al*, 1997; Rice-Evans,1995). Part of the antioxidant activity may also be due to tannins, which are astringent antioxidants and known to occur in *Abies, Picea, Tsuga, Thuja, Juniperus, Nuphar, Quercus, Populus, Gaultheria, Dirca, Rhus, Prunus, Sorbus,* and *Smilacina* (Arnason *et al.*, 1981).

Antioxidant Vitamins

Vitamin C (ascorbic acid) is a strong dietary antioxidant. Fourteen percent of the plants sampled here where statistically similar to vitamin C (ascorbic acid) in free radical scavenging, 63% in superoxide scavenging and 97% in peroxyl scavenging. Some of these native plants have been examined for vitamin content in relation to their use as food

with vitamin A or C reported from 34 % of the selected species (Kuhnlein and Turner, 1991).

Vitamin E is a dietary antioxidant which has been investigated in regard to its affect on diabetes (Reaven *et al.*, 1995; Cotter *et al.*,1995; Salonen *et al.*,1995; Paolisso *et al.*,1993). Trolox®, a water-soluble analogue of vitamin E, is a positive control of antioxidant activity in these experiments. In our experiments we found 23% of the tested plants responded significantly better than Trolox® in free radical scavenging, 29% were similar to it in superoxide scavenging and 60% were similar to it in peroxyl scavenging. The positive comparison of these plants to vitamin E in regard to antioxidant activity bodes well for their potential use as antioxidant agents for diabetics.

It has been suggested that the combined antioxidant activity of the two dietary antioxidants vitamin E and C is greater than their individual actions (Niki *et al.*, 1995; Thomas *et al.*,1995; Cotter *et al.*,1995). Many of the plants tested herein likely have, in addition to antioxidant vitamins, the added antioxidant benefit of tannins and phenolic mixtures.

Comparison to Tea

Tea, especially green tea, has been studied extensively in relation to its antioxidant activity. The antioxidant activity of tea has been considered in research against cancer (Katiyar and Mukhtar,1997) and cardiovascular disease (Tijburg *et al.*,1997). In addition green tea (Matsumoto *et al.*,1993) and black tea (Gomes et al,1995) have shown antidiabetic activity in the reduction of blood glucose. The extensive research in tea's antioxidant activity in relation to these diseases suggests potential uses, and future studies, of the plants reported here which have similar antioxidant activity. Eight boreal forest species have similar free radical scavenging activity in comparison to green tea and three have similar superoxide scavenging (Tukey analysis, $P<0.05$). Several tea studies have used the DPPH radical assay (Gadow *et al.*, 1997a, Nanjo *et al.*, 1996) and tea catechins have been tested in superoxide assays (Yen and Chen, 1995, Noda *et al.*, 1997). Our assays confirm the high antioxidant

activity of green tea as reported in the literature as well as greater antioxidant activity of green tea than trolox® or ascorbic acid.

Black tea has also shown good antioxidant activity, but to a lower degree, than green tea. This is probably a factor of the fermentation process that reduces catechin content to 9% from green tea's 30% (Wiseman *et al.*, 1997). In this study the activity of black tea in relation to lettuce, carrot and potato is consistent with other reports (Cao *et al.*, 1996).

Preparation of any tea product, as well as additions before consuming, should be considered when reviewing data from various sources and methods. The length of brewing time (Gadow *et al.*, 1997b) or the addition of milk can have a significant effect on the antioxidant activity of tea *in vivo* (Serafini *et al.*, 1996).

Tea is known to contain tannin, with most of its antioxidant activity attributed to catechins (Nanjo *et al.*, 1996). Rather then being a single chemical, tea has the combined activity of flavonoids, most being catechins, theaflavins and flavonols (Wiseman *et al.*, 1997), that can lead to enhanced activity. The medicinal plants tested in this paper have this combined potential as well.

Comparison to Wine and Coffee

Coffee had relatively low antioxidant activity. Against free radicals 46% of the medicinal plants performed significantly better than coffee. Against superoxides and peroxyl radicals 37% and 57% of the plants, respectively, had greater mean activity than coffee although *Rhus hirta* was the only plant to have significantly greater activity in both assays (Tukey analysis, $P<0.05$). Interestingly, some studies on humans have found coffee to have a slight detrimental effect on glucose tolerance (Goldman and Ovadia, 1969; Jankelson *et al.*, 1967). Consumption of antioxidant-containing plants, such as *Rhus hirta,* as tonics or teas may indeed be more beneficial than coffee to individuals with symptoms of diabetes.

Wine has been publicized for its antioxidant activity and in relation to the "French Paradox"-a case of lower coronary heart disease than predicted from a high fat diet (Kinsella *et al.*,1993). The wine we tested had significantly lower free radical scavenging activity than 83% of the medicinal plants tested with 20% of the plants also higher in superoxide scavenging. Only one of the medicinal plants had a lower mean peroxyl scavenging activity. Such low activity is most likely due to the nature of the wine purchased- a mixed blend for the table. The type of wine can make a difference in antioxidant activity (Frankel, *et al.*,1995). A cabernet sauvignon would have yielded a higher result due to its tannins while a white wine, with lower anthocyanin content, would likely have yielded a lower result. Tannins and anthocyanins could also be factors in the antioxidant activities found in medicinal plants.

Comparison to market produce

Most of the medicinal plants (89%) had free radical scavenging activity significantly greater than the market produce tested. Twenty-nine percent had significantly greater superoxide scavenging activity than the market produce, which also did not perform well against peroxyl radicals. The great difference between green tea versus garlic and the other market produce is also evident in the work of Cao *et al.* (1996) who showed, in an ORAC assay using AAPH, green tea to be over 20 times greater in antioxidant activity than garlic, potato, carrot and iceberg lettuce. Garlic (Table 3) also performed comparatively poorly in our antioxidant assays. The most likely explanation for garlic's low activity is that extract preparation can significantly affect garlic's active principles (Tyler, 1993). The type of plant, collecting protocol and sample preparation can all affect the final plant product. All the plants and market produce were prepared in the same way- dried and powdered before extraction.

Although carrots contain carotenes, the antioxidant activity of this vegetable was significantly lower than many of the plants tested here. This does not discount the importance of market produce as a source of antioxidants in the diabetic diet. Dose, and quantity in the diet are important criteria for overall antioxidant contribution.

Tannin's effect on health and assays

Tannin itself is a strong antioxidant and is an especially prominent component in some of these plants (i.e. *Rhus hirta* and *Quercus* sp.). Some of the known affects of tannin are considered to be detrimental to health. Tannins combine with proteins (Haslam,1996) or make iron more unavailable. However, the addition of ascorbic acid (possibly also contained in these plants) can prevent the inhibition of iron absorption (Siegenberg *et al.*,1991). Tannins have the potential to cause liver damage but are also postulated to have beneficial effects in digestion, the ability to detoxify some plant chemicals and action against tooth decay (Johns, 1990). Concerns about toxicity can be somewhat alleviated considering the quantities consumed in tea are likely minute, however, toxicological studies would be advantageous. The tannin content should not rule out these plants as potential treatments considering the benefits afforded by the antioxidant activity. In addition tannin could have possible combined or synergistic effect with other antioxidants within the plant extract which may be a factor in its activity.

Since tannins are known to bind to proteins they are of concern in many laboratory assays involving enzymes. The only experiment used here that employed an enzyme that might be argued to be affected by tannin is the superoxide assay using hypoxanthine and xanthine oxidase. However, Hatano *et al.* (1990) concluded that any inhibition by tannin in this system is indeed due to antioxidant activity and not action upon the enzyme.

Because of the above concerns plant extracts are often detannified prior to experimental procedures. However, until more is known about the mixed chemical nature of these extracts, no single component should be discounted as not contributing to the total beneficial effect. For this reason the extracts were not detannified. Promising extracts could indeed be further evaluated without the tannins in order to identify other antioxidant components and the total percentage of antioxidant action attributed to the tannins.

Conclusions

Some of the indigenous plant treatments of diabetes or its complications contribute antioxidants to the traditional lifestyle in greater amounts than is currently available via the market produce and beverages tested and some to a greater degree than standard antioxidant vitamins. Since antioxidants have been found to be beneficial in the prevention of the complications of diabetes this study supports and validates the use of these traditional medicines.

As the use of these traditional plants decrease in relation to changes in lifestyles, people likely reduce their ingestion of antioxidants that could prevent complications of diabetes. Plants ingested in a traditional lifestyle often do not easily conform to food, beverage or medicine catagories (Johns, 1990). Considering that 57% of these species are used as tonics and 60% of these plants are also known to be used for food as well as medicine (Kuhnlein and Turner, 1991), their consumption may represent a significant contribution of antioxidants.

These results suggest that the plant treatments of the Indigenous Peoples living within the Canadian boreal forest warrant further testing in the areas of prediabetes and antioxidant bioavailability. Studies on hypoglycemic activity, cholesterol lowering capacity, absorption and direct effects on such symptoms as retinopathy are definitely needed.

INTRODUCTION TO MANUSCRIPT B

Following the determination of antioxidant activity in these extracts questions remain regarding variation in the levels of antioxidants within the plants collected. Oxidative stress can occur in plants (Dalton, 1995; Mishra *et al.*, 1993; Hagan *et al.*, 1993) and as such determine the amount of antioxidants in a given part of a plant subjected to those stresses. Traditional medicinal plants that are specifically used in regard to a particular plant tissue may be a way of selecting from the varying amounts of compounds that can occur within the different parts of a plant. In the following manuscript we have evaluated the medicinal plants reported in manuscript A in relation to growth condition and habit as well as overall selection of plant part and preparation of these traditional medicines.

MANUSCRIPT B

The importance of traditional herbalist selection of plant parts and species growth habit on antioxidant activity of medicinal plant species.

Letitia M. McCune and Timothy Johns

ABSTRACT

Considering that oxidative stress is important in diabetes and the complications of diabetes, 35 medicinal plants used for the treatment of symptoms of diabetes were evaluated previously for antioxidant activity (McCune and Johns, manuscript A). In this paper we evaluate the affect of plant part, habitat, growth structure and preparation techniques on the level of antioxidant activity of these medicinal plants. The more frequently used parts of the plant used medicinally were roots and barks. Activity of the bark extracts averaged to 21.38 +/-3.84 ppm while roots had an activity of 185.11+/-32.18 ppm in a free radical DPPH assay. In contrast the analysis of overall parts (medicinal or not) in these species found leaves and barks to have the least activity (105.91+/-29.91 ppm and 108.83 +/-19.64 ppm, respectively). The highest activity was found in trees (24.88 +/-3.32ppm) as compared to herbs and shrubs, and increased activity was found in habitats of decreased water/fertility. No significant differences were found over all the means in comparing standard methanol extracts to those of water. Our findings scientifically verify the selection and preparation of these plant remedies in relation to antioxidant activity.

Keywords: part, plant extracts, methanol, tea, habit, habitat, medicinal

INTRODUCTION

Environmental factors such as UV stress, insect damage and competition can determine the antioxidants present in a plant (McKey, 1979; Polle and Rennenberg, 1993; Larson, 1988). Growing conditions and preparation methods of medicinal plants could also affect the antioxidant properties of an herbal remedy. Delineation of patterns of presence of antioxidants in medicinal species can assist in the identification of potentially useful products as well as provide insight into the manner in which traditional indigenous healers make selective decisions about herbal remedies.

Oxidative stress has been linked to many diseases, and antioxidants are increasingly promoted in the scientific and popular literature as beneficial components in the treatments of many conditions (e.g.. Halliwell, 1997; Packer and Colman, 1999). We have recently analyzed the antioxidants in traditional plant remedies from the Canadian boreal forest used by Indigenous Peoples for symptoms of diabetes and its complications (McCune and Johns, manuscripts A and C). Of the latter retinopathy, atherosclerosis and peripheral neuropathies are some of the conditions associated with oxidative stress (Baynes, 1991; Giugliano *et al.*, 1995;Van Dam and Bravenboer, 1997; Thompson and Godin, 1995) . By identifying those plants used for three or more of the symptoms of diabetes or its complications we have found a high rate of antioxidant activity among the selected medicinal plants.

Different parts of a plant derive different benefits from accumulating phenolics or other types of antioxidant phytochemicals. Such compounds are important in pollinator recognition patterns on petals, as light or UV protectants, as feeding deterrents in leaves and bark and as allelopathic compounds in leaves, roots and other tissue (Polle and Rennenberg, 1993; Harborne, 1991; McKey, 1979). Thus selection of the antioxidant activity of medicinal remedies by traditional herbalists may be at the level of a specific plant part. Considering that many of the factors associated with antioxidant accumulation are related to growing conditions, analysis of medicinal species in relation to antioxidant

activity and growth habitat could provide insight into the likely location of species high in antioxidant activity.

In addition, by analyzing the traditional methods used to prepare medicinal species for disease symptoms associated with oxidative stress we can better assess scientifically the traditional gathering practices of these medicinal species. The analysis of 35 medicinal species used by Indigenous Peoples of the boreal forest of Canada presented here concentrates on traditional collection practices related to the plant part used, growth conditions of the medicinal species and herbal preparation that could contribute to the antioxidant activity of a plant remedy.

METHODS

Species collection and detection of antioxidant activity

Medicinal plant species were identified by literature review. They encompass those plants used by the Indigenous Peoples of the North American boreal forest for three or more symptoms of diabetes or its complications. They were collected in the months of June through September in the years 1996 and 1997 as described in McCune and Johns (manuscript A). Antioxidant activity was determined through free radical scavenging activity detected using 1,1 diphenyl-2-picryl-hydroazyl (DPPH) as prevously reported (McCune and Johns, manuscript A). The activity presented is inversely related to the value. The numbers represent the concentration of extract (ppm) that reduced the standard oxidant by half (IC_{50}) as originally determined with ascorbic acid. The lower the concentration required and reported for this reduction, the greater the activity. In all cases antioxidant activity was concluded from at least three independent experiments performed in duplicate.

Extraction technique

Ten medicinal species were selected at random in order to compare the methanol extraction method used in routine analysis and water extractions that are closer to traditional preparation techniques. Methanol extraction was performed on samples air-dried in the shade, ground and extracted in a Soxtec extractor as previously described

(McCune and Johns, manuscript A). Ten grams of powdered sample was extracted with 210 ml methanol over a period of 3 hours.

Water extraction was adapted from the work of Sanchez de Medina *et al.* (1994) and achieved through a combination of infusion and decoction. Twenty grams of powdered sample was added to 40 ml of distilled water for 5 minutes. This was then followed by another 160 ml, gentle mixing for 10 minutes and filtration. The residue was further extracted for 15 minutes with 200 ml boiling water before filtration. In the third stage the residue was extracted by simmering for 15 minutes in 200 ml of water and then filtered. The three water extracts were pooled before lyophilization. When the samples were fresh frozen they were finely chopped before extraction.

Statistics

Tukey analysis was performed on the means of the DPPH antioxidant activity [using a transformation of log(y+1)] for the primary parts used from each medicinal plant, the parts of randomly selected medicinal species, growth habit and growing condition using a SAS® statistical computer program. A Students' t test was used to determine statistically significant differences among parts of the same species and between different extracts of the same species.

RESULTS

The number of recorded species for which fruit, bark, leaves or roots respectively were used medicinally are present in Table 1. Roots and bark/twigs were used the most frequently (13 and 12 species, respectively) followed by leaves with 9 species. Although only one fruit was identified (*Rhus hirta*), the greatest free radical scavenging activity was found in this fruit (3.73 +/- 0.07 ppm), with bark/twigs second (21.38 +/- 3.84 ppm), leaves third (31.16 +/- 3.55 ppm) and roots a distant last (185.11 +/- 32.18 ppm). All of the means for the different species parts were significantly different (tukey analysis).

The differences between plant parts that may or may not have been used medicinally are presented in Table 2. Three or more different parts per species were evaluated from a

Table 1. Antioxidant activity of parts used medicinally

part	species number	activity (IC_{50}) ppm +/- S.E.			significance[1]
fruit	1	3.73	+/-	0.07	a
bark/twigs	12	21.38	+/-	3.84	b
leaves	9	31.16	+/-	3.55	c
roots	13	185.11	+/-	32.18	d

[1] means with the same letter are not significantly different [tukey analysis of log(y+1)]

Table 2. Analysis of all parts available from 10 randomly selected medicinal species (species with more than 3 parts tested)

part	species number	activity (IC_{50}) ppm +/- S.E.			significance[1]
flowers	4	35.04	+/-	4.98	a
fruit	2	36.98	+/-	15.23	b
roots	11	75.26	+/-	11.25	c
leaves	11	105.91	+/-	28.91	d
bark/twigs	7	108.83	+/-	19.64	e

[1] means with the same letter are not significantly different [tukey analysis of log(y+1)]

random selection of 10 of the 35 medicinal species. There were more roots and leaves (11), than bark/twigs (7), fruit (2) or flowers (4) available for analysis. Fruit and flowers were highest in activity (35.04 +/- 4.98 ppm and 36.98 +/- 15.23 ppm, respectively). Roots had intermediate activity (75.26 +/- 11.25 ppm) while leaves and bark/twigs had the least activity (105.91 +/- 29.91 ppm and 108.83 +/- 19.64 ppm, respectively).

Table 3 directly compares the activity (IC_{50} in DPPH tests of free radical activity) of the parts of the 10 species randomly selected (as used in Table 2) from the 35 medicinal plant species. The part used medicinally is presented in the first column while the other columns list other parts tested within the same species. Statistical differences were observed (t-test) between the other parts within the same species and the medicinal part, except in *Rhus hirta* roots and *Solidago canadensis* flowers. In six of the eleven species tested the medicinal part had statistically greater activity (lower IC_{50}) than at least one other part, while 5 species (*Achillea millefolium, Acorus calamus, Asarum canadensis, Dirca palustris* and *Populus tremuloides*) had less activity in their medicinal part as compared to the other parts tested.

Table 4 groups the medicinal species into categories of growth habit. More of the medicinal species could be described as trees (14), followed by herbs (12) and shrubs (9). The highest antioxidant activity (24.88 +/- 3.32 ppm) was found in the tree group followed by shrubs and then herbs. All three of the groupings were considered significantly different in antioxidant activity as judged by Tukey analysis.

Table 5 separates the 35 different medicinal species tested into general categories based on the habitat or growing condition. Those plant species growing in wet or boggy areas had a mean activity of 118.94 +/- 30.94 ppm which was significantly less than those growing in woodland and rocky or infertile areas (76.74 +/- 20.06 ppm and 40.82 +/- 5.18 ppm, respectively).

The antioxidant activity found in the water extracts derived from fresh frozen or dried samples and that obtained from methanol extracts of dried material is compared in Table

Table 3. Comparison of antioxidant activity in the medicinally used part to other parts of the same species

species	Activity (IC_{50}) ppm +/- standard error											
	medicinal part[1]			other parts								
Achillea millefolium	**67.64**	+/-	2.75(r)	**41.23**	+/-	2.80(f)**	**35.86**	+/-	1.65(l)**			
Acorus calamus	**75.71**	+/-	5.89(r)	**53.17**	+/-	1.56(l)**	**52.35**	+/-	3.23(f)**			
Aralia racemosa	**152.10**	+/-	5.44(r)	**291.38**	+/-	4.70(s)**	**579.39**	+/-	58.08(l)**			
Asarum canadensis	**87.78**	+/-	2.82(r)	**70.24**	+/-	7.38(f)*	**33.47**	+/-	1.76(l)**			
Celastrus scandens	**91.33**	+/-	3.84(r)	**74.46**	+/-	4.25(b)*	**138.09**	+/-	2.29(l)**			
Dirca palustris	**233.28**	+/-	7.94(r)	**149.24**	+/-	0.94(b)**	**161.22**	+/-	7.54(l)**			
Nuphar varigatum	**6.02**	+/-	0.59(r)	**23.32**	+/-	1.17(l)**	**9.92**	+/-	0.64(f)**			
Populus balsamea	**42.81**	+/-	4.64(l)	**62.11**	+/-	1.66(r)**	**23.68**	+/-	0.39(b)**			
Populus tremuloides	**102.90**	+/-	4.06(b)	**7.19**	+/-	0.22(r)**	**36.91**	+/-	3.51(l)**			
Rhus hirta	**3.73**	+/-	0.07(fr)	**4.77**	+/-	0.60(r)	**6.72**	+/-	0.64(s)**	**5.44**	+/-	0.21(l)**
Solidago canadensis	**42.51**	+/-	3.83(r)	**36.66**	+/-	6.44(f)	**55.32**	+/-	4.64(l)*	**113.39**	+/-	2.50(s)**

[1] r=roots,l=leaves,b=bark,f=flowers,s=stems,fr=fruit

* and ** are significant differences (t-test) determined at P<0.05 and P<0.01, respectively, in comparison to the medicinal part of the species

Table 4. Growth habit and antioxidant activity of medicinal species

growth habit	species number	activity (IC_{50}) ppm +/- S.E.			significance[1]
herbs	12	179.54	+/-	34.87	a
shrubs	9	48.65	+/-	13.33	b
trees	14	24.88	+/-	3.32	c

[1] means with the same letter are not significantly different [tukey analysis of log(y+1)]

Table 5. Comparison of antioxidant activity in medicinal species according to growing condition

habitat	species number	activity (IC_{50}) ppm +/- S.E.			significance[1]
bogs or wet areas	11	118.94	+/-	30.94	a
woodland	16	76.74	+/-	20.06	b
rocky or infertile	8	40.82	+/-	5.18	b

[1] means with the same letter are not significantly different [tukey analysis of log(y+1)]

6. No significant differences over all the means were determined between the two methods (Tukey analysis). Of the 10 randomly selected medicinal species *Achillea millefolia* roots and *Celastris scandens* roots were the two species that exhibited the greatest increase in activity in the methanol extract compared to that of the water. Significant differences in the order of less than 11ppm were found in the activity achieved from the two methods for four other species, *Corylus cornuta, Juniperus virginiana, Nuphar varigatum* and *Taxus canadensis*.

DISCUSSION

The data presented here support the supposition that selection and preparation of traditional medicinal plants has an important effect on antioxidant activity. Individual parts of a species selected for use against symptoms of diabetes and its complications have different levels of antioxidant activity from other parts of the species or from those parts selected at random. The growing conditions of the medicinal species were also shown to have an affect on antioxidant activity. The traditional method of preparation was found comparable to methanol extraction for the recovery of antioxidant activity.

Naturally-occurring plant antioxidants are most commonly phenolics (Pratt, 1992). These compounds contain at least one aromatic ring with hydroxyl groups, and encompasses, among others, the tannins, coumarins, flavonoids (including isoflavonoids, anthocyanins, catechins, chalcones, flavones and flavonols) and lignins (Strack,1997). In some cases flavonoids have been found to have greater antioxidant activity than trolox or ascorbic acid (Cao *et al.*,1997). Most of these compounds are important to the plant in protective roles as secondary metabolites. Considering that the array of phenolics includes a vast number of compounds (there are over 4000 flavonoids alone), and that many of these compounds are unique enough to be used in chemosystematics to distinguish one species from another (Seigler, 1981;Cao *et al.*, 1997), there could be any number of compounds causing the antioxidant activity differences between species.

Table 6. Comparison of antioxidant activity in water extracts to methanol extracts[1]

species	water extract		methanol extract
	dried	fresh frozen	dried
***Achillea millefolia* roots**	**143.5** +/- 2.34**	**158.30** +/- 7.37**	**68.20** +/- 3.30
***Celastris scandens* root**	**278.35** +/- 11.06**	**158.10** +/- 10.40**	**91.33** +/- 3.85
***Corylus cornuta* bark**	**13.15** +/- 0.88**	**11.81** +/- 0.71**	**8.82** +/- 0.23
***Juniperus communis* bark**	**10.16** +/- 1.33	**16.51** +/- 2.74	**11.82** +/- 2.33
***Juniperus virginiana* leaves**	**29.23** +/- 1.23	**40.68** +/- 1.91*	**29.76** +/- 2.47
***Nuphar varigatum* roots**	**9.89** +/- 0.29**	**8.14** +/- 0.77*	**6.02** +/- 0.59
***Picea mariana* bark**	**17.72** +/- 1.56	**24.00** +/- 0.57	**17.19** +/- 2.80
***Prunus serotina* bark**	**18.61** +/- 1.14	**17.63** +/- 1.07	**17.78** +/- 3.93
***Rhus typhina* fruit**	**3.90** +/- 0.12	**3.94** +/- 0.24	**3.73** +/- 0.07
***Taxus canadensis* leaves**	**61.66** +/- 2.88*	**52.54** +/- 5.55	**51.09** +/- 3.63

[1] reported in ppm (IC_{50}) +/- standard error

* is a significant difference ($P<0.05$) from the methanol extract (t-test)

** is a significant difference ($P<0.01$) from the methanol extract (t-test)

Note:tukey analysis determined no significant difference overall between extraction techniques

1. Effect of Plant Part

Our results demonstrate the difference in antioxidant activity among the various parts of medicinal plants. Tables 1, 2 and 3 show that the part selected for medicinal purposes has significantly different antioxidant activity from another part of the same species randomly selected. The differences could be due to phenolics, including flavonoids, which can fluctuate with the growth of a plant or the stresses it must respond to in order to survive. In identifying species based on chemical compounds (chemosystematics), it is known that compounds vary between different parts of a plant (Seigler, 1981). This is especially important to be aware of in the use of medicinal plants in the herbal medicine market as it is not unheard of to find different parts used for the same medicinal product (Newall *et al*, 1996). This could cause the consumption of different compounds or quantities of compounds than were originally intended.

1a. Bark

Bark made up over a third of the plant parts used medicinally and were second only to the single sample of fruit in antioxidant activity (Table 1). Because bark was lowest in activity when sampled at random (Table 2), this suggests a possible selection of barks higher in antioxidants. Antioxidant activity could be due to, among others, catechins, tannins or lignins. Barks or woods used for foods and spices have been found to contain more catechins than other flavonoids (Pratt, 1992). Tannins and lignins are common components of barks. Tannins are known to act as feeding deterrents and lignins as support in the conducting tissues of wood (Hagerman and Butler, 1991).

Dufour and Wilson (1994) found the barks of trees and vines among those plants used by the Amerindians were known to contain higher amounts of chemical defenses. In addition, the bark or sap of trees or vines account for 75% of the medicines of the Baka pygmies of W. Africa (Lewington, 1990). Chemical defenses could include compounds (e.g. tannins) that are high in antioxidant activity making these barks promising medicinal products.

1b. Leaves

The activity of the leaves used for medicinal purposes was close in activity to that of the bark. There is a highly significant difference (t-test, P=0.006) between the activity of the leaves selected at random and those used medicinally (106 ppm vs. 31 ppm, respectively). This again suggests selection for activity.

Leaves are known to have a greater variety of flavonoids than barks and their amounts can fluctuate during seasonal changes, although the relative amounts of different flavonoids are constant (Niemann, 1988). Flavonoids such as anthocyanins, as well as antioxidant terpenoids like carotenoids, that become apparent in the leaves when foliage colors change in the fall are thought to protect the photosynthetic machinery from UV stress. Studies on tobacco supplemented with UV light found a two fold increase in flavonoids and phenolics (Anderson and Kasperbauer, 1971).

Phenolics in leaves can also play a role in allelopathy when upon decomposition on the ground toxic water soluble compounds can limit the nearby growth of other plants (Strack, 1997). Flavonoids involved in such defenses include quercetin, myricetin, phloretin, catechin and epicatechin (Stafford, 1990).

1c. Flowers and fruits

The highest activity of the randomly selected parts was found in the flowers. Antioxidants can be present in flowers in the form of anthocyanins and carotenoids for petal color and pollinator recognition patterns (Ho, 1992). However, flowers were not used medicinally in any of the 35 species identified from the literature. The low use of flowers could be due to the presence of unpleasant tasting compounds (evolved to limit foraging of reproductive structures) or allergens (i.e.pollen) found in flowers. In addition, the ephemeral nature of flowers would allow only a short time period for collection thereby limiting their use for practical reasons.

Although fruits selected randomly from the medicinal plants was second only to flowers in activity among the parts tested (Table 2), their activity is still lower than the one fruit actually used for medicinal purposes (Table 1). Although the number of species was low, at 2 and 1 respectively, the high activity in these plants is intriguing. Fruit, especially those designed for consumption in order to disperse seeds, would contain fewer toxins or feeding deterrents but still have the array of colorful antioxidant attractants. The seeds present in fruit may also contain antioxidants. Coumarins, a type of phenolic antioxidant, have been found to be important in seed dormancy (Salisbury and Ross, 1992). As well, vitamin E, a dietary antioxidant present in rice hulls, has been found to be important to the longevity of the grain (Ramarathnam *et al.*, 1989). The low use of fruit may be due to the relatively short duration of most fruits through the year, whether a result of animal consumption or decay.

1d. Roots

Of the plant parts used medicinally, roots had the lowest antioxidant activity. Over one third of the total medicinal species were used for the roots but the random analysis of roots from the medicinal species yielded a higher activity (75 ppm vs. 185 ppm). There are several reasons the roots could be selected for lower antioxidant levels.

The roots in Table 1 may have been selected from medicinal plants with specific toxins present in the aerial portions of the plant at greater quantities than in the root of the species. Chemical defenses including toxins against insects and animal browsers may be present at higher concentrations in those parts of certain plants more easily reached by certain browsers- i.e. leaves and bark. Roots with low antioxidant activity seems, however, more the exception than the rule as the antioxidant activity of roots in the random selection was higher than both leaves and bark (Table 2).

Roots, could also have been deliberately selected from certain species in order to gain an oxidative, rather than an antioxidant, benefit for certain symptoms. Lin *et al.* (1995) discuss the possibility that Chinese plant medicines used for anti-inflammatory reasons have antioxidant capabilities while those used as antiviral or antimicrobial agents have

increased superoxide production capabilities. While 80% of all the medicinal species we selected had better antioxidant activity than a 100 ppm IC_{50}, the roots accounted for 86 % of those with lower activity than 100 ppm (see manuscript A). Most of those roots were used for symptoms that included sores/wounds and abcesses/boils-conditions that would require antimicrobial agents. Roots low in antioxidant activity could therefore have been selected from certain species for their low toxins and/or superoxide antimicrobial activity as well as for possibly other unknown medicinal compounds.

There are also ecological reasons that could account for the antioxidant activity present in the roots in Table 2. One would expect some antioxidant activity in roots due to the need for chemical defenses against soil pathogens and insects. Indeed, phenolics are known to be excreted by some roots in order to reduce competition or to promote nitrogen-fixing rhizobacterium association (Strack,1997). A strong root structure is important to the plant for stability and survival through the years and therefore worth defending with chemical defenses that may be antioxidants.

2. Effect of Growth habit

The results presented in Table 4 suggest that trees and shrubs (totaling 23 species) were more often used as medicinal plants than herbs (12). Moerman (1994) in reviewing his compilation of general plant uses in North America also found a greater percentage of trees and shrubs were used medicinally than expected from total plants used and grouped into categories of growth habit. Other than the arguments in favor of antioxidant activity in barks, as discussed above, woody species would more likely allow for continued collection of bark or leaves (evergreens) throughout the year. Considering their smaller stature and seasonal above ground presence herbs also might be harder to locate than woody species. This seasonal presence may account, in part, for the fact that for over 90% (11 out of 12) of the herbaceous medicinal species it was their roots that were collected for medicines. In addition when collecting some herbaceous plants (e.g.. species from the genus *Aralia*) it is easier to collect a large

amount of aromatic roots from one plant than it is to harvest the same amount of above ground portion. This ease of access argument may also account for the greater use of bark over leaves or fruit as barks and roots would be more available throughout the year.

These same arguments can be applied to perennials as compared to annuals. In fact, none of the medicinal species of this study were annuals. Only one species (*Verbascum thapsus*-a biennial) was not considered a perennial. Considering their greater need for chemical defenses in order to survive through the years, perennials would be considered better choices when searching for phenolics then annuals.

3.Effect of Habitat

Those species located in woodland or rocky/infertile areas had greater activity (lower IC_{50}) than those of wet or boggy areas. This may be due to the increased UV stress in the sunnier, drier areas, leading to increased flavonoid protection. Considering the boreal forest by definition is more forest than wet areas and bogs (Department of Mines and Technical Surveys, 1957), it is not surprising that most of the medicinal species are from woodland areas. Collecting species from wet and boggy areas would require extra effort and caution thereby contributing to lower collections in those areas. However, despite these obstacles these wet area species were collected. Interestingly, most of these species were used for symptoms (e.g. boils/abscesses, swelling, skin problems and sore or swollen limbs) associated with low antioxidant activity (McCune and Johns, manuscript C) thereby suggesting possible oxidant production (Lin *et al.*, 1995). In addition, when we analyzed symptoms by growing condition of the medicinal species (Tukey not shown), we found these symptoms to be grouped at 1.6 and below (1=bogs, 2=woodland, 3=rocky). These wet area plants may have distinct properties worth the extra collecting effort.

4.Effect of Sample Extraction Method

We have shown here that the traditional method of preparation (i.e. tea) is comparable, in antioxidant activity, to a standard phytochemical extraction with methanol of these

particular species. Phenolics, including flavonoids, are mainly water-soluble and are often associated with the cell vacuole and chloroplasts. In some cases, water has proven to be the best extractant for antioxidant activity in medicinal plants (Filipek, 1994). Extraction, however, is often carried out with alcohol in order to ensure that no enzyme degradation occurs (Harborne, 1973) and to obtain compounds with the greatest range of solubilities. Two of the randomly selected species, *Achillea millefolia* roots and *Celastris scandens* roots, had a difference in water extract activity greater than 11ppm and significantly different than in the methanol fraction. Perhaps these two have greater amounts of degrading enzymes in their tissue. Differences between the two methods of the four other species were in the order of less than 11ppm.

In addition we have shown there was little difference between the water-extracted samples taken from fresh frozen or dried specimens. Often in phytochemical analysis the fresh samples have higher general activity than the dried samples. Any decrease in activity in a dried sample can be a result of tannins being bound to other parts of the plant cells, chemical degradation or volatility (Harborne, 1984). The only decrease greater than 10ppm we found was in the *Celastris scandens* root. Considering that all parts of this plant are potentially toxic (Foster and Duke,1990), a greater amount of extraction of compounds may not be desired from this species.

There are numerous criteria for selecting the best extraction method. Yield of beneficial over noxious compounds, biohazard of extractant itself and ease of extraction are some important criteria. Eloff (1998) used these criteria to analyze various extractants of two plant species shown to have antimicrobial activity. He found acetone to be the best extractant because of its ease of handling and low toxicity in his bioassay involving bacteria. However, as we are evaluating the preparation of medicinal plants for human consumption, we give a higher value to water in regard to its low toxicity and take more into account the higher levels of extraction found. Eloff (1998) found the highest level of extraction in his plants was achieved with water and therefore our experiments agree with Eloff's and we conclude the traditional water method appears the best for human consumption purposes.

Selection Practices

Selection practices in relation to plants selected for medicinal reasons have not been broadly researched. The best analysis we know of was performed by Moerman on his compilation of plants used by the Indigenous Peoples of North America (Moerman, 1996). This is a large database of 44,775 items on native North American plant use. Although not related to a specific activity as reported here, he did find trees and shrubs were used more frequently for medicinals than could be predicted, that most medicinals were herbs and a greater percentage were perennials than annuals. Our look at 35 species for a particular set of symptoms found the majority of medicinals attributed to herbs and trees with less found as shrubs. None of the species used in this study were annuals. He also separated medicinal plants according to plant part used, unfortunately he grouped bark, leaves and branches as "plant top" together to compare to fruit and root. Plant top and root he found to be used the most frequently for medicinals while fruit use was much lower. We as well found few uses for fruit for our selected symptoms and more use of bark and root.

Each study of medicinal plants used by traditional herbalists in a given part of the world is influenced by the diseases associated with living in that area as well as the plants available for collection. The results here specifically deal with those plants used for the symptoms of diabetes and its complications and used by traditional herbalists of the boreal forest of Canada.

Conclusions

The differences we have found in antioxidant activity in the parts of selected medicinal plants suggest a possible selection by traditional herbalists for those parts high in antioxidant activity. Our results also imply that when collecting plants for antioxidant activity that one should look in woodland and rocky/infertile areas over areas that are boggy or wet. In addition selection of perennial plants was highly favored as was trees and shrubs over herbaceous species. Traditional preparations of these medicinal remedies

in the form of teas was also found to be comparable in antioxidant activity to those of a standard phytochemical method involving methanol .

Factors that could not be addressed in this work include the effect of seasonal variation, the age of the plant, population variations and specific harvesting techniques. Minimum variation due to these factors was expected considering the species were harvested in the same growing season, from single populations and prepared in the same manner. It would be interesting to continue investigating these medicinal species in traditional gathering sites to determine any differences in antioxidant activity.

Our findings suggest that the traditional methods employed by the Indigenous Peoples of the Canadian boreal forest in the selection and preparation of these medicinal plants could be a means of increasing the input of antioxidant activity into the traditional lifestyle. Further studies comparing these plant remedies to the general flora not used medicinally could augment these conclusions.

INTRODUCTION TO MANUSCRIPT C

Knowing that these medicinal plants have antioxidant activity, and that certain collection practices appear to increase the chance of antioxidant activity in the medicinal preparation, one begins to wonder if the reason for the collection (i.e. the specific symptoms related to the treatment) is related to the level of antioxidant activity. Determining the relationship between antioxidant activity and symptom would better equip a pharmacognosist in hunting for plants with high activity based on the ethnobotanical use of the plant. The following manuscript reports the specific symptoms used by each of the medicinal plants studied in the previous manuscripts and uses statistical techniques to group the activities based on number of symptoms used per species, according to each symptom individually and via groups of specific symptoms.

MANUSCRIPT C

Diabetic symptoms related to antioxidant activity in some traditional plant remedies

Letitia M. McCune and Timothy Johns

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ABSTRACT

Previous research analyzed the antioxidant activity in 35 medicinal plant species used for 3 or more symptoms of diabetes or its complications (McCune and Johns, manuscript A). In this report we study the link of antioxidant activity to 21 specific symptoms, the number of symptoms treated by a species and clusters of symptom use per species. The results revealed that the greater the number of symptoms treated by a species (i.e. over 5 symptoms) the greater the antioxidant activity of the species with the least activity in those species used for 4 symptoms. The top 6 symptoms associated with high antioxidant activity were sexual irritability, diarrhea, rheumatism/arthritis, tonic, heart/chest pain and urinary conditions. The symptoms associated with lowest activity included swelling, abscesses/boils, general medicine and sores/wounds. The literature gives examples of many of these symptoms associated with high antioxidant activity being treated with antioxidants and many of the lower symptoms treated with oxidants. Clusters of symptoms found to be associated with high antioxidant activity included diarrhea and heart/chest pain within a species and tonic, sores/wounds, urinary, blood purifier, pregnancy and abscess/boils. This study gives scientific validation to ethnobotanical records related to symptoms of diabetes and to those species used for numerous symptoms.

INTRODUCTION

The many diseases and medical problems associated with oxidative stress include those of the diabetic complications (Halliwell and Gutteridge, 1990). Oxidative stress and diabetes has been the subject of several reviews (Oberley, 1988; Baynes 1991; Giugliano *et al.*,1995) and antioxidants can therefore be of benefit in the treatment of these diseases. Vitamin E in particular has been studied in regard to the treatment of heart disease and arteriosclerosis (Stephens *et al.*, 1996; Stampfer and Rimm, 1995) as well as cataracts and retinopathy (Fryer, 1993).

Among the numerous symptoms associated with diabetes itself are sugars in the urine, fatigue and increased thirst. The complications of the disease can include infections of the urinary system and vagina, kidney malfunctions, peripheral neuropathies (including sexual dysfunction) and infections leading to amputations, eye problems including retinopathy and cataracts and angiopathy. The leading cause of death among diabetics is atherosclerosis, a form of angiopathy. In addition other predictors of diabetes include difficult pregnancies, intestinal upsets and headache (Zeman and Hansen, 1991).

Worldwide 151 million people are estimated to suffer from diabetes in the year 2000, with the number increasing to 221 million by the year 2010 (Amos *et al.*,1997). Indigenous Peoples of Canada are 3-5 times as likely to get this disease, principally type 2 diabetes (or non-insulin dependant diabetes mellitus), than the overall population (Canadian Diabetes Association, 1998; Young *et al.*,1990). A genetic factor, specifically a "thrifty gene", is suggested to allow for rapid conversion of energy in times of plenty to protect life during famine (Diamond,1992).

Accounts of similar medical conditions to diabetes are found in literature dating over 2000 years ago (Bailey and Day, 1989) in conjunction with records of plant treatments. Recently, a review of the NAPRALERT database found 1200 species of organisms to have been used for diabetes (Marles and Farnsworth, 1995), 81% of the 295 traditional

plant remedies tested in this study lowered blood glucose. We propose that such plants should also be analyzed for antioxidant activity.

There are several good reviews of literature on medicinal plant use by the many different groups of Indigenous Peoples within Canada (Helm,1981; Kuhnlein and Turner, 1991) and North America (Arnason *et al.*,1981; Erichsen-Brown, 1979; Hutchens, 1991; Moerman, 1998). In conducting our literature search we concentrated on those peoples living within the boreal forest and on uses of medicinal plants for numerous symptoms of diabetes. We then proceeded to select those species used for 3 or more symptoms of diabetes or its complications. Similar ethnobotanical or ethnomedical techniques have been recently discussed in the search for new antidiabetic drugs (Oubre *et al.*,1997; Carlson *et al.*, 1997).

We previously reported on the antioxidant activities of 35 different selected plant species (McCune and Johns, manuscript A). In this paper we will expand on that work by concentrating on each symptom itself and the number of symptoms each plant species was used for in relation to diabetes. In this manner we hope to highlight the importance of antioxidants in the treatment of some of these conditions as well as the ethnobotanical/medical approach to drug or treatment discovery for this prevalent disease.

METHODS

Plant species

Plant species used by Indigenous Peoples of the boreal forest region of Canada for 3 or more symptoms of diabetes or its complications were selected for study (McCune and Johns, manuscript A). These symptoms are listed at the bottom of Table 1. Specimens were collected in single populations in the vicinity of Montreal, Quebec, Canada as reported previously (McCune and Johns, manuscript A). Voucher specimens are housed in the McGill University herbarium (Ste. Anne de Bellevue, Quebec).

Table 1. Plants used for the treatment of the symptoms of diabetes and its complications

Species used medicinally (common name), voucher ID	# diabetic symptoms treated	specific symptoms[1]	Tonic/Beverage(b) food(f),vitamin a/c (v)[2]	Indigenous Peoples[3]
Abies balsamea bark/twigs (balsam fir), 18	12	ABCDEFHIJKLQ	bfv	Ojibwa, Algonquin, Montagnais, Micmac
Achillea millefolium flowers (yarrow), 15	5	DAMNO	b	Ojibwa, Algonquin, Cree
Acorus calamus roots (sweet flag), 24	5	PMQKD	b	Iroquois, Ojibwa, Algonquin, Malecite, Cree
Aralia nudicaulis roots (wild sarsaparilla), 7	8	HKPESIDR	b	Ojibwa, Algonquin, Abenaki, Montagnais
Aralia racemosa roots (spikenard), 19	5	HCEAI	f	Ojibwa, Micmac, Malecite
Arisaema triphyllum roots (indian turnip), 3	3	CMH		Ojibwa, Malecite
Asarum canadense roots (wild ginger), 12	4	ONMA	f	Ojibwa, Montagnais
Celastrus scandens roots (climbing bitter-sweet), 8	5	MHESV		Iroquois, Ojibwa
Cornus stolonifera bark (red-osier dogwood), 9	5	CFMEA	b	Ojibwa, Algonquin, Abenaki, Malecite
Corylus cornuta bark/twigs (beaked hazelnut), 28	3	FCQ	fv	Algonquin, Abenaki, Cree
Dirca palustris root bark (moosewood), 20	4	EVMB		Iroquois, Ojibwa

Table 1. *continued*

Species used medicinally (common name), voucher ID	# diabetic symptoms treated	specific symptoms[1]	Tonic/Beverage(b) food(f),vitamin a/c (v)[2]	Indigenous Peoples[3]
Gaultheria procumbens leaves (wintergreen), 13	5	IRJAM	fb	Iroquois, Ojibwa, Algonquin, Cree
Heracleum lanatum roots (cow parsnip), 23	3	EHML	fv	Ojibwa, Micmac, Cree
Juniperus communis bark (juniper), 30	7	JEVHDSI	bfv	Micmac, Malecite, Cree, Delaware
Juniperus virginiana leaves/twigs (red cedar), 31	3	JAV		Ojibwa, Cree
Kalmia angustifolia leaves/flowers (sheep laurel), 11	7	IJANLDF	b	Montagnais, Micmac, Malecite, Cree
Ledum groenlandicum leaves (Labrador tea), P. Owen #15	8	HDKARVIE	bfv	Ojibwa, Algonquin, Montagnais, Micmac, Malecite, Cree
Nuphar variegatum roots (yellow water-lily), 32	4	PLWM	f	Iroquois, Algonquin, Abenaki, Micmac
Picea glauca bark/twigs (white spruce), 17	7	JSRMVEA	bfv	Ojibwa, Algonquin, Montagnais, Abenaki, Micmac, Cree
Picea mariana leaves (black spruce), 16	6	DIASRE	bv	Ojibwa, Algonquin, Cree
Populus balsamifera buds (balsam poplar), 27	7	EQPISOR	f	Ojibwa, Algonquin, Micmac, Malecite, Cree
Populus tremuloides bark (quaking aspen), 14	5	EXQJH	bfv	Ojibwa, Cree, Abitibi

Table 1. *continued*

Species used medicinally (common name), voucher ID	# diabetic symptoms treated	specific symptoms[1]	Tonic/Beverage(b) food(f),vitamin a/c (v)[2]	Indigenous Peoples[3]
Prunus serotina bark (rum cherry), 22	5	RHEQD	bfv	Iroquois, Ojibwa, Malecite, Delaware
Quercus alba bark (white oak), 33	4	FVBD	bf	Ojibwa, Micmac, Malecite, Delaware
Quercus rubra bark (red oak), 6	3	QMF	f	Ojibwa, Malecite
Rhus hirta fruit (staghorn sumac), 4	6	VKEDFR	bfv	Iroquois, Algonquin, Malecite, Delaware
Sassafras albidum bark (sassafras), store purchase	4	JVKR	bf	Iroquois, Ojibwa
Smilacina racemosa roots (false spikenard), 2	5	AIKES	f	Ojibwa, Abenaki, Algonquin
Solidago canadensis roots (goldenrod), 29	7	HQVBEKD	bf	Ojibwa
Sorbus americana bark (mountain ash), 25	6	MRKHIS	f	Ojibwa, Algonquin, Montagnais, Malecite, Cree
Taraxacum officinale roots (dandelion), 1	3	IRK	bfv	Iroquois, Ojibwa, Algonquin, Cree
Taxus canadensis twigs/leaves (yew), 26	5	JKSNF	b	Ojibwa, Algonquin, Montagnais, Abenaki, Micmac, Malecite
Thuja occidentalis leaves (arbor vitae/white cedar), 10	9	KAIBJQLMX	bv	Iroquois, Ojibwa, Algonquin, Montagnais, Abenaki, Malecite, Micmac

Table 1. *continued*

Species used medicinally (common name), voucher ID	# diabetic symptoms treated	specific symptoms[1]	Tonic/Beverage(b) food(f),vitamin a/c (v)[2]	Indigenous Peoples[3]
Tsuga canadensis bark (hemlock), 5	6	EFBKJI	bfv	Ojibwa, Algonquin, Abenaki, Malecite, Micmac
Verbascum thapsus roots (mullein), 21	3	LEQ		Iroquois, Ojibwa, Malecite

[1] A=headache, B=skin, C=sore eyes, D=reviver/tonic, E=sores/wounds, F=diarrhea, H=abcesses/boils, I=back pain/kidneys, J=rheumatism/arthritis, K=childbirth/ pregnancy, L=swelling, M=general medicine/physic, N=pain, O=inflammation, P=circulation, Q=heart /chest pain, R=blood purifier/blood tonic, S=fainting/weakness (including anemia), V=urinary/diuretic, W=sexual irritability, X=sore or swollen limbs.

[2] As described in Arnason *et al.*, 1981 and Kuhnlein and Turner, 1991 for selected Indigenous Peoples.

[3] Listed is only those Indigenous Peoples within the area of Canada that used the species for diabetes related symptoms as listed in Arnason *et al.*,1981

Antioxidant activity

Collections were air dried in the shade. Material was ground in a Wiley mill to a particle size of 850μm and extracted with a Soxtec® apparatus (HT2, 1045 extraction unit) with methanol (HPLC grade) at a rate of 10g/210 ml. Extracts were stored at -20°C in teflon lidded amber jars. Free radical scavenging activity was determined via a spectrophotometer assay using 1,1 diphenyl-2-picryl-hydroazyl (DPPH). Superoxide scavenging potential was analyzed via hypoxanthine/xanthine oxidase and nitroblue tetrazolium (assay labelled "XO" in tables). Peroxyl radicals were produced by an azo intiator [2,2'-diazobis(2-amidinopropase)dihydrochloride or AAPH] and scavenging analyzed with 2,7-dichlorofluorescin-diacetate (DCF). Methods and results of these assays were reported previously (McCune and Johns, manuscript A).

Statistical methods

The SAS® statistical computer program was used for all statistical analyses. Multicomparison analysis of the symptoms was done using the Tukey method at $P<0.05$. The model was y = species(symptom) + symptom. Cluster analysis employed the 'average' cluster procedure that created differences between binary data sets created for the symptoms associated with each species. In order to limit the chaining and/or individual species clusters 7 species were removed via the "TRIM" and "kth nearest neighbor" procedure.

RESULTS

Species list

The 35 species that were analyzed in relation to their antioxidant activity (McCune and Johns, manuscript A) are listed in Table 1 with the 21 different symptoms used in their selection. The 3 or more symptoms of diabetes or its complications for which each species are used are included in the second column. From the literature we found 37 % of these species have known quantities of the antioxidant vitamins A and/or C and 63%

of these species are also known to be used for foods and 60% for beverages or tonics. The Indigenous Peoples of the boreal forest region of Canada who used the species as described are represented in the last column.

Antioxidant activity associated with specific symptoms

The symptoms of diabetes and its complications are listed in Table 2 in order of decreasing DPPH free radical antioxidant activity. The lower the mean value the greater the activity as judged by IC_{50} concentrations comparable to the action of ascorbic acid in the antioxidant assay. Symptoms of heart disease, diarrhea, urinary issues, circulation and blood disorders as well as species used for a reviver or tonic are all below values of 60. In contrast abcesses or boils, sores or wounds and general medicine had lower activity. Superoxide activity is listed according to the XO assay. Higher values in this case represent higher activity according to % inhibition. Tukey multicomparison analysis places circulation, inflammation and pain as symptoms associated with a lower rank of activity in the XO superoxide scavenging assay as compared to the DPPH assay. The Tukey analysis of antioxidant activity according to the DCF assay found only sexual irritability and weakness/fainting to be significantly different in peroxyl radical scavenging although the general means in this assay ranked pregnancy higher and sore limbs lower than the DPPH assay. A Kruskal-Wallis rank test applied to the ranks of the 3 antioxidant assays found there were significant differences between the different assays.

Symptom number

Table 3 lists mean values associated with groups of species being used for different numbers of symptoms. Species used for 7 or 12 symptoms had values in the low 20's while those used for only 4 or 5 symptoms had lower activity with means over 100. Using Tukey analysis species used for symptoms over 5 in number has significantly greater activity than those used for 3,4 or 5 symptoms. The same trend (i.e. greater symptoms, greater activity) is shown for superoxide scavenging (XO) with the exception of the low outlier of 8 symptoms. Peroxyl radical scavenging (DCF) had the same trend but no values were shown to be statistically different via Tukey analysis.

Table 2: Average antioxidant activities according to the symptom use of the medicinal species

Symptom	number of species*	antioxidant activities (mean +/- S.E.)**		
		DPPH (IC_{50} in ppm)	XO (% inhibition)	DCF (% inhibition)
sexual irritability	1	6.0 +/- 0.6 a***	24.3 +/- 1.7 a	18.9 +/- 0.5 a
diarrhea	10	15.4 +/- 2.4 ab	26.4 +/- 1.9 a	18.0 +/- 1.5 ab
rheumatism/arthritis	11	30.1 +/- 4.3 bc	16.5 +/- 1.5 bcd	14.8 +/- 1.1 ab
reviver/tonic	14	30.8 +/- 3.9 bc	17.6 +/- 2.1 bc	18.5 +/- 1.3 ab
heart/chest pain	11	39.8 +/- 5.2 cd	14.8 +/- 1.5 bcdefg	16.8 +/- 1.3 ab
urinary issues	10	47.7 +/- 11.9 cde	17.7 +/- 2.7 b	18.3 +/- 1.7 ab
circulation	4	50.9 +/- 9.1 cde	7.2 +/- 2.9 h	14.7 +/- 1.5 ab
pain	4	55.5 +/- 7.9 de	11.7 +/- 2.2 efgh	13.0 +/- 1.9 ab
blood purifier/tonic	12	60.5 +/- 15.2 de	12.9 +/- 2.0 cdefg	14.5 +/- 1.3 ab
inflammation	3	66.2 +/- 6.1 e	7.4 +/- 0.8 h	13.4 +/- 1.1 ab
sore or swollen limbs	2	67.3 +/- 16.1 efg	12.3 +/- 2.5 defg	11.8 +/- 1.5 ab
headache	14	79.9 +/- 24.0 fgh	13.3 +/- 1.6 bcdefg	13.2 +/- 1.1 ab
skin	7	86.1 +/- 22.7 ghi	17.4 +/- 2.6 bc	15.4 +/- 1.9 ab
sore eyes	5	86.7 +/- 27.2 ghij	15.5 +/- 3.6 bcdef	13.5 +/- 1.7 ab
back pain/kidneys	15	93.4 +/- 24.6 hij	13.4 +/- 1.6 bcdefg	12.4 +/- 1.1 ab
childbirth/pregnancy	14	98.3 +/- 26.5 hijk	13.7 +/- 2.0 bcdefg	15.2 +/- 1.4 ab
weakness/fainting	9	102.2 +/- 35.6 hijkl	10.6 +/- 2.0 gh	11.4 +/- 1.5 b
sores/wounds	20	109.2 +/- 23.3 ijkl	13.8 +/- 1.8 bcdefg	14.3 +/- 1.2 ab
general medicine/physic	14	109.5 +/- 24.8 jkl	11.7 +/- 1.7 efgh	12.9 +/- 1.0 ab
abcesses/boils	12	117.6 +/- 27.6 kl	10.8 +/- 1.8 fgh	14.0 +/- 1.4 ab
swelling	6	122.0 +/- 51.9 l	15.8 +/- 2.3 bcde	12.7 +/- 1.6 ab

*the number of medicinal species of the 35 tested that were used for the indicated symptom

**means and standard errors of three different antioxidant assays on medicinal species averaged according to symptom use

***means with the same letter within an assay are not statistically different as determined by tukey analysis

Table 3: Antioxidant activity according to the number of symptoms treated by the tested medicinal species

# symptoms	number of species[1]	antioxidant activities (mean +/- S.E.)[3]		
		DPPH (IC_{50} in ppm)	XO (% inhibition)	DCF (% inhibition)
12	1	**22.2** +/- 1.1 a[2]	**22.5** +/- 0.4 a	**17.5** +/- 1.3 a
7	4	**25.6** +/- 4.0 a	**19.3** +/- 2.2 ab	**20** +/- 2.2 a
9	1	**31.6** +/- 2.2 a	**17.3** +/- 2.5 ab	**10.8** +/- 2.6 a
6	6	**37.5** +/- 13.3 a	**20.8** +/- 3.7 a	**15.6** +/- 2.4 a
8	3	**46.4** +/- 11.6 a	**5.3** +/- 3.3 d	**15.1** +/- 2.7 a
3	5	**83.3** +/- 31.0 b	**14.4** +/- 2.1 bc	**14.4** +/- 1.5 a
5	9	**106.4** +/- 31.9 b	**9.7** +/- 1.9 cd	**12** +/- 1.3 a
4	6	**208.9** +/- 52.5 c	**11.2** +/- 3.4 c	**11.6** +/- 1.6 a

[1]the number of the 35 medicinal species tested that were used for the number of symptoms indicated

[2]tukey multicomparison analysis, means with the same letter within an assay are not statistically different

[3]means of three different antioxidant assays on medicinal species averaged according to multiple symptom use

Cluster analysis of symptoms

Seven clusters of symptoms were identified and listed in Table 4. They are presented in order of mean DPPH activity of the species within the cluster. The clusters of symptoms labeled 1 through 4 have activities of 20 ppm (IC_{50}) or below.

DISCUSSION

Our results indicate that selection of plant species for such symptoms as diarrhea, tonic, heart disease or urinary conditions can lead to a selection of plants with high levels of antioxidant activity. In addition the number of symptoms a plant is used for in relation to diabetes can also indicate the level of antioxidant activity. In analyzing our data by cluster analysis we also found certain groups of symptoms treated by one species can signify high antioxidant levels.

Specific symptoms and antioxidant activity

Most of the symptoms we found to be associated with plants of high antioxidant activity are also known to be treatable with antioxidants. Antioxidants in plants encompasses a wide variety of compounds including flavonoids, phenolic acids, chlorophyll derivatives, enzymes, carotenoids, vitamins (including ascorbic acid, or vitamin C, and vitamin E) and tannins (Larson, 1988; Pratt, 1992). Phenolics (including flavonoids) in particular are often the main components of antioxidant levels in plants (Pratt, 1992; Lindhorst, 1998) and are known to have some additional pharmaceutical benefits (Pein-Gen and Shan-Lin, 1985; Middleton, 1985).

The highest antioxidant activities were found in those plants used for symptoms of diarrhea whether studied singly (Table 2) or grouped with heart ailments (Table 4). Diarrhea or intestinal upsets are sometimes treated with plants high in tannins (Haslam, 1996). Tannins, in addition to being strong antioxidants, are known to have astringent properties involving the binding of protein. This can lead to a soothing or protective coat being produced in the intestine to protect irritated mucosal cells (Haslam, 1996).

Table 4: Cluster analysis according to symptom use

cluster[1]	group of symptoms	species included	mean (IC_{50} in ppm)[2]
1	diarrhea, heart/chest pain	*Corylus cornuta, Quercus rubra*	10.2 +/- 1.4
2	headache, rheumatism/ arthritis, general medicine/physic blood purifier/blood tonic	*Gaultheria procumbens, Picea glauca*	18.0 +/- 1.6
3	reviver/tonic, sores/wounds urinary/diuretic, blood purifier/blood tonic, pregnancy abscesses/boils	*Juniperus communis, Prunus serotina, Rhus hirta, Sassafras albidum, Solidago canadensis*	20.0 +/- 6.6
4	back pain/kidneys, rheumatism/arthritis, headache, skin, diarrhea, pregnancy, swelling	*Abies balsamea, Kalmia angustifolia, Thuja occidentalis, Verbascum thapsis*	20.6 +/- 4.3
5	headache, sore eyes, reviver/tonic, sores/wounds	*Achillea millifolium, Asarum canadensi*	77.7 +/- 10.1
6	back pain/kidneys, blood purifier/blood tonic, pregnancy, sores/wounds, headache, abscesses/boils, faint/weak	*Aralia nudicaulis, Aralia racemosa, Ledum groenlandicum, Picea mariana, Smilacea racemosa, Sorbus americana, Taraxacum officinalis*	179.2 +/- 86.8
7	sores/wounds, general medicine/physic	*Celastris scandens, Dirca palustris, Heracleum lanatum*	316.9 +/- 159.9

[1]clusters based solely on symptoms (10 species were considered unique and not grouped)

[2]means +/- standard errors are based on DPPH data

Heart problems (coupled with chest pain) as a symptom was associated with considerable antioxidant activity (Table 2), and with diarrhea produced the cluster of symptoms highest in activity (Table 4). Heart disease and arteriosclerosis can both cause fatigue and shortness of breath thereby leading one to take a "tonic" or reviver (another symptom associated with high antioxidant activity in Table 2). These conditions are related to blockage of the arteries often associated with lipid peroxidation of circulating LDL and/or arterial wall membrane lipids. Lipid peroxidation has been extensively studied recently in relation to treatment with antioxidants- especially vitamin E (Traber and Sies, 1996; Stampfer and Rimm, 1995). The antioxidant activity of green tea flavonoids has also been studied in relation to cardiovascular diseases (Tijburg *et al.*, 1997). It is interesting to note the closeness of the mean antioxidant values for heart disease, circulation and blood problems (Table 2).

Urinary problems were also coupled with high activity in Table 2 and 4. Urinary issues are sometimes treated with antioxidants. Ascorbic acid as well as cranberry juice is frequently suggested as a treatment of urinary infections. Although originally thought to act by increasing the acidity of the urine, cranberries are effective by decreasing the binding of bacteria to the bladder wall through the action of proanthocyanidins (Howell *et al.*, 1998). Proanthocyanidins in the highly antioxidant blueberry were also found to limit the binding of bacteria. Cranberry extract is also known to contain amounts of the antioxidant gallic acid in the amounts of 1,548mg/l (Wilson *et al*, 1998). In addition the progression of chronic renal failure has been known to decrease with a treatment of antioxidant via green tea tannin (Yokozawa *et al.*, 1996).

The symptom category arthritis/rheumatism had high antioxidant activity in Table 2 as well as clustering in some symptom groupings in Table 4. Treatments for arthritis and rheumatism have recently been investigated in relation to antioxidants action (Eugui *et al.*, 1994) as well as with certain plant derived compounds (Meloni *et al.*,1995; Kang *et al.*,1996). In these studies tumor necrosis factor (TNF) production is reduced thereby potentially decreasing inflammation of the joints. Because TNF is also linked to insulin

resistance we have investigated the affect of the better antioxidant producers of our tested plant species on TNF production in a human cell line (McCune and Johns, manuscript D).

Symptoms with low antioxidant activity

On the other end of the range of antioxidant means we found relatively low activity for such symptoms as abcesses and boils, sores and wounds and use as a "general medicine". General medicines could refer to those plants that boost the immune system. TNF is known to stimulate the immune system and considering that certain antioxidants can decrease the TNF production of macrophage (Eugui *et al.*, 1994), it follows that one may want this low antioxidant activity when fighting an infection. In addition, in contrast to antioxidants, oxidants (e.g.. hydrogen peroxide lotions) are commonly used to fight infections in the over-the-counter pharmaceutical market. The plant medicines with low antioxidant activity may have other activities as well that were not addressed in this study.

Some of the symptoms had few plant species associated with them. This is seen in the case of sexual irritability. However, the results are still worth mentioning considering this symptom is related to peripheral neuropathies in the diabetic which has been linked to oxidative stress (Cotter *et al.*,1995; Van Dam and Bravenboer, 1997). The low sample sizes of some of the symptoms may add to the difference between the Tukey analysis ranking and the numerical order of the means. This does not seem to have affected the discussed over all ranking of symptoms.

Number of symptoms and grouping of symptoms per species

Because antioxidants are important in so many of the complications of diabetes it follows that individual medicinal plants used for many of these different symptoms could have antioxidant activity. Indeed we have found that the greater the number of symptoms a species was used for the greater was the general free radical antioxidant activity of the species. Our results suggest when searching for species with high antioxidant free radical activity one should look for those species with greater than 5 of the listed symptoms related to diabetes. For 8-12 symptoms the activity (IC_{50}'s below 50) was statistically similar (Tukey analysis) whereas species with 4 symptoms as a group has the lowest activity.

Different types of antioxidant activity varied in their levels of significance in a group of species. For those species high in activity against superoxides, the general trend was similar to that of the free radicals with greater activity in those with more than 5 symptoms although species with 8 and 5 symptoms had activity below that of those with 3 symptoms. The peroxyl radical scavenging results (DCF) did not differentiate between species based on number of symptoms (Tukey analysis). Individual results on 12 and 9 symptoms for any of the assays should be interpreted with caution considering these groupings have only one species each.

Recognizing that the greater the number of symptoms the greater the activity, the question then arises what combination of symptoms is best. Using cluster analysis we found 4 groupings of symptoms (#1-4 in Table 4) to have activities of 20 ppm or below. It is interesting that these groupings have such good activity with low standard error considering the selection was based purely on symptoms. The greatest activity was found in the group of species (containing *Corylus cornuta* and *Quercus rubra*) used for diarrhea and heart symptoms (among others). A greater number of species (5) were found to be used for the symptoms listed in cluster 3 (tonic, wounds, urinary, blood, pregnancy and abscesses), including *Rhus hirta* and *Solidago canadensis*. In addition to having been used for a similar grouping of symptoms these two species we found to have some of the greatest general activities of the species tested (McCune and Johns, manuscript A).

Pregnancy, sores/wounds and skin conditions were the three symptoms that fluctuated the most between the different assays. Overall they moved up in rank the most in assays DCF and XO as compared to DPPH (Table 2). Interestingly, these same symptoms appear as important symptoms in the clusters of high activity.

The clusters we named 5, 6 and 7 had lower activity (higher mean ppm). Cluster 6 in particular has a wide range of activities (13.6ppm – 624.4ppm) among its member species (data not shown). Therefore we cannot suggest this grouping of symptoms for searching species for antioxidant activity.

CONCLUSIONS

Medicinal plants were an integral part of the traditional lifestyle. As witnessed here these plants would have contributed antioxidants that would have helped control many of the complications that may have arisen from fluctuating sugars in the blood present in a prediabetic. Those plants also used for tonics, beverages or food were more likely taken in greater amounts over time then for plants we generally consider for medicines and this pattern of ingestion would thereby increase the overall intake of antioxidants. The antioxidant vitamin E has been studied in reducing the onset of diabetes (Salonen *et al.*,1995). Perhaps practices that enhanced antioxidant ingestion in the traditional lifestyle were a factor in keeping the incidence of diabetes lower than the rates of today.

General antioxidant assays could easily be incorporated into high throughput screening of plants for medicinal properties. However, an integral part of this use for diabetic symptoms is the link to symptoms as well as the use of one species for more than one symptom, thus supporting the use of ethnobotanical data for the selection of plant species for potential drug discovery. A further analysis of medicinal plants for numerous symptoms could include the evaluation of plants not used medicinally to determine the general antioxidant status of plants used for less than 3 symptoms or in the environment in general. This study however does give scientific validation to the ethnobotanical records of use of these plant medicines for certain symptoms of diabetes and its complications, as well as the use of one species for numerous symptoms.

INTRODUCTION TO MANUSCRIPT D

The previous manuscripts have found levels of antioxidant activity in a number of the medicinal plant extracts and have linked them to the use of these for specific symptoms of the complications of diabetes. What remains is to show an affect of these extracts in a diabetic model. Examination of hypoglycemic action in a diabetic model, although interesting, would not have emphasized the antioxidant capabilities on diabetes. Any hypoglycemic action in this case would have been attributed to unknown compounds in these plants and not necessarily the antioxidant compounds. Considering that many of the artificial means used to create diabetes in many diabetic models are easily affected by antioxidants, we searched for another means of evaluating antioxidant affects on diabetes. In addition, if we did use a diabetic model such as the ob or db mouse, we would need to evaluate the extract affects on all of the symptoms of diabetes or its complications (e.g., heart and cardiovascular disease, cataracts, renal and urinary problems, ischemic injury and nerve damage, etc.). This would have been time consuming and expensive. However, if we had concentrated on only one specific complication of diabetes we would not have appropriately represented the plants' traditional use as these plants are used for multiple symptoms of diabetes.

Considering that NIDDM is characterized as a disease of insulin resistance, we selected a model that specifically dealt with this aspect of diabetes. TNF is linked to insulin resistance (Hotamisligil *et al.*, 1994; Kahn, 1995) as well as increased oxidative stress (Goossens *et al.*, 1995). By studying the affects on TNF by those plant extracts highest in antioxidant activity we hoped to be able to determine a potential link between these species and an alleviation of symptoms related to diabetes and its complications. Considering the laboratory facilities available the human macrophage *in vitro* model was used to study TNF production as it was determined relatively easy to obtain and study. It is used extensively in the literature and is a means of studying the effects in a human rather than a rodent model.

MANUSCRIPT D

Effect of *Rhus hirta, Cornus stolonifera* and *Solidago canadensis* extracts on tumor necrosis factor-α production in human macrophage cells

Letitia M. McCune and Timothy Johns

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ABSTRACT

Rhus hirta fruit (*Rh*), *Cornus stolonifera* roots (*Cs*) and *Solidago canadensis* bark (*Sc*) were selected for this study on the basis of their traditional use for symptoms of diabetes and its complications as well as their previously determined high antioxidant activity (McCune and Johns, manuscript A). We present here the effect of the methanolic extracts of these plants on tumor necrosis factor-α (TNF) production in U-937 human macrophage cell cultures. Cell viability, tannin and pH affects were examined. *Cs* inhibited TNF production the most with 44.68% +/- 11.38 at 0.1μg/ml and 36.73% +/- 0.99 at 0.01μg/ml. *Sc* followed with 30.89% +/- 7.62 at 1μg/ml and *Rh* with 29.13% +/- 5.94 at 0.01μg/ml. In an experiment comparing all extracts at 10 μg/ml *Sc* showed the greatest inhibition. Green tea, black tea, coffee and ascorbic acid stimulated TNF production and Trolox® and lettuce had little effect. The phenolic antioxidants in these extracts likely inhibit TNF production thereby contributing to effects on the symptoms of diabetes.

Key words: TNF, extracts, diabetes, antioxidant, plant

INTRODUCTION

Tumor necrosis factor-α (TNF) can cause insulin resistance as well as an increase in reactive oxygen species leading to the complications of diabetes. Insulin resistance can be caused by TNF reducing insulin receptor tyrosine kinase activity (Hotamisligil *et al.*,1994; Kahn, 1995) as well as affecting the GLUT4 glucose transporter (Stephens and Pekala, 1991). TNF levels are increased in non-insulin–dependent diabetes mellitus (NIDDM) and there have been some findings of increased levels of TNF in prediabetics (Hussain, 1996).

TNF circulates through the body in the bloodstream. It is produced in excess by adipocytes and its interaction with free fatty acids is a possible link between obesity and diabetes (Hotamisligil and Spiegelman, 1994; Boden, 1997). Oxidative stress is involved in many of the complications of diabetes including atherosclerosis (the leading cause of death among diabetics), nephropathy, neuropathy, cateracts and retinopathy (Halliwell and Gutteridge, 1990). TNF can increase reactive oxygen species and therefore oxidative stress, although the exact mechanism is largely unknown, (Goossens *et al.*,1995). TNF has been linked to many diabetic complications (Nakamura *et al.*,1998a; Hawrami *et al.*, 1996; Kluth and Rees, 1996). As well as increasing reactive oxygen species, TNF has the property of increasing inflammation (Dinarello, 1997).

Some plant derived compounds or extracts used for symptoms of arthritis or immune deficiency have been investigated for their ability to decrease TNF production (Meloni *et al.*,1995; Kang *et al.*,1996; Chang *et al.*,1995). Interestingly, Kang *et al.* isolated a diterpene from a plant used for diabetes as well as rheumatism. Because some known antioxidants such as quercetin, BHA (butylated hydroxyanisole), THP (tetrahydropapaveroline), apomorphine and NDGA (nordihydroguaiauretic acid) inhibit TNF production (Eugui *et al.*,1994; Sato *et al.*,1997), recent studies have focussed on potential antioxidant treatments for inflammation via the inhibition of TNF production. Since TNF is produced by macrophages, an *in vitro* experiment involving human macrophages and plant extracts used for diabetic complications and known to have antioxidant activity, can be used to screen potential treatments. Experiments of this sort could then support absorption, toxicological, hypoglycemic or specific diabetic complication studies *in vivo*.

We have recently researched various plant treatments of diabetic symptoms or complications in relation to antioxidant ability (McCune and Johns, manuscript A). Three of the plant treatments (*Rhus hirta*, *Cornus stolonifera* and *Solidago canadensis*) were chosen as the highest in antioxidant activity based on experiments of free radical, peroxyl and superoxide radical scavenging. The crude extracts of these plant species are examined here to determine if they also decrease TNF production as a further benefit in the treatment of diabetic symptoms. Traditional uses for *Rhus hirta* include treatments for frequent urination, wounds, birth, tonic, purification of blood and diarrhea; for *Cornus stolonifera* they include sore eyes, diarrhea, general medicine, stoppage of bleeding and headache; and for *Solidago canadensis* uses are heart disease, stoppage of urine, difficult labor and boils.

Considering that these remedies are normally taken in the form of a water infusion (tea) we have included some common beverages (green tea, black tea and coffee) for comparative purposes. Green tea is of current interest for the prevention of cancer (Ahmad and Mukhtar, 1999) and recent studies demonstrate inhibition of TNF production by epigallocatechin gallate (EGCG), the primary flavonoid and antioxidant of green tea (Yang *et al.*, 1998). Ascorbic acid and Trolox® (a water soluble form of vitamin E) have been included as standard antioxidants. In contrast to the antioxidants, lettuce extract was included as a negative control as it did not perform well in the previous antioxidant tests.

METHODS

Extract preparation

Rhus hirta fruit and *Solidago canadensis* roots were collected from single populations in Ile Perrot, Quebec Canada (45° 23′N, 73° 57′ W) and *Cornus stolonifera* bark was collected in Ste. Anne de Bellevue, Quebec Canada (45° 25′N, 73° 56′ W). *Rhus hirta* was collected in July, 1996; *Cornus stolonifera* in June, 1996 and *Solidago canadensis* in August, 1997. Voucher specimens are housed in the McGill University herbarium. Samples were air dried in the shade before grinding in a Wiley mill to a particle size of

850μm. Samples represent a collection of plants (≤ one kg of dried material) from a single population. Green tea (Twinings brand), black tea (Red Rose brand), coffee (Maxwell House brand) and iceburg lettuce were purchased from a local supermarket. The lettuce was freeze-dried prior to grinding in the mill. Extractions were performed in methanol (HPLC grade) using a Soxtec® extractor (HT2, 1045 extraction unit) at the rate of 10g of powdered sample to 210ml methanol over a period of 3 hours. Methanol and moisture were removed via rotoevaporation and lyophilization. The concentrated extracts were stored at –20°C in amber jars with teflon-lined caps. L-Ascorbic acid and Trolox® standards were obtained from BDH and Sigma respectively.

Cell culture

U937 human monocyte cells, originally established by Sundstrom and Nilsson (1976)(ATCC), were cultured in RPMI -1640 medium containing 10 mM HEPES, 1mM sodium pyruvate, 2mM L-glutamine, 4500 mg glucose/L, and 1500 mg sodium bicarbonate/L (ATCC) and supplemented with 10% (v/v) fetal calf serum (Sigma). Differentiation into mature macrophage was achieved by incubating the cells with phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich Canada) in multiwell plates (Falcon brand, Fisher) at a concentration of 10^{-9}M PMA /10^{6}cells/ml for 48 hours (Wang et al.,1997). Cells were washed 2 times with Hanks balanced salts (Sigma) followed by an incubation of 24 hours in RPMI. Stock solutions were prepared of the extracts and standards for each experiment. The extracts and standards were dissolved in RPMI with the help of a sonicator bath, filtered and added to the differentiated cells after a dilution series in RPMI. In order to stimulate TNF-α production, lipopolysaccharide (LPS from E. coli, serotype 0111:B4, Sigma) at a final concentration of 1.0μg/ml was added at the same time as the extracts (Meloni *et al.*,1995). Control production of TNF was determined from cell cultures stimulated with LPS. After 24 hours supernatants were removed from the cell cultures, centrifuged and analyzed via a human TNFα ELISA kit (R&D Systems).

Statistics

All experiments were performed at least three times in duplicate. Results represent averages ± the standard error. Statistical analysis was performed using ANOVA and paired Student's t tests.

RESULTS

Cell viability tests using trypan blue dye exclusion determined that for most of the extracts cell death was no different than the controls (data not shown). This was evident in an ANOVA analysis of all extracts used at 10μg/ml. However, at 100μg/ml *Rhus hirta* increased the cell death significantly to 5% greater than the controls, and *Solidago canadensis* significantly to 7% greater than the controls (t-test, $P<0.01$). There was no significant increase in death rate among the concentrations tested with *Cornus stolonifera.* Addition of the extracts did not affect the pH of the cell media and tannin was found not to influence the readings from the ELISA kit (data not shown).

Inhibition of TNF production at 10μg/ml: A comparison of extracts

Figure 1 illustrates the TNF production of the cells stimulated with LPS upon the addition of 10μg/ml of extract. All three of the plant treatment extracts (*Rhus hirta*, *Cornus stolonifera* and *Solidago canadensis*) produced an average decrease in TNF with the addition of Solidago causing a highly significant inhibition of 19.88 ± 3.76% at 10 μg/ml (t-test, $P<0.01$). The standard antioxidants produced either a significant stimulation of TNF (ascorbic acid, 23.33% ± 9.24) or a slight inhibition (Trolox®, 2.11% ± 6.20). Lettuce, as the negative control to antioxidants (as determined in McCune and Johns,manuscript A), produced a slight inhibition of 3.17% ± 3.86. The beverages, black tea and coffee, produced some stimulation of TNF production while stimulation by green tea was significant at 21.82% ± 9.98 ($P<0.05$).

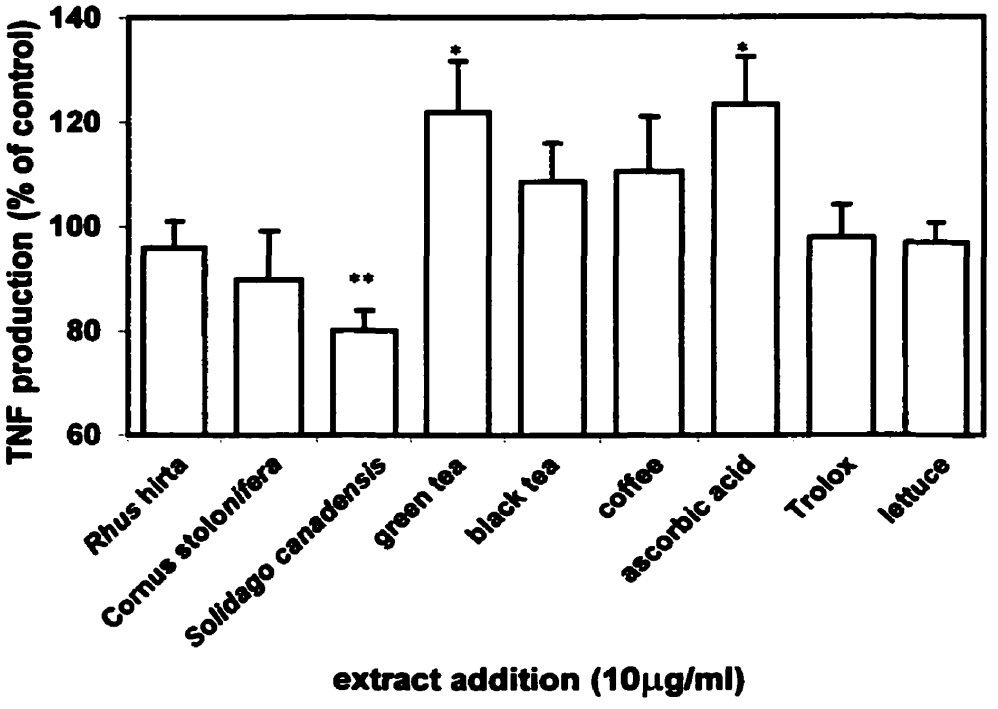

Figure 1. The comparison of extract or antioxidant standard additions on TNF production in U937 human macrophage cells stimulated with LPS. The control, as 100%, is the amount of TNF produced from the macrophage cells stimulated with LPS. The data are expressed as mean +/- standard error (t-test in relation to control,*P<0.05, **P<0.01).

Inhibition at different doses

Rhus hirta showed a highly significant inhibition as compared to the control (t-test, $P<0.01$) at 0.01 μg/ml followed by a gradual lessening of inhibition until a stimulation of TNF was produced at 100 μg/ml (figure 2). As represented in figure 3 a similar pattern emerged with *Cornus stolonifera*. *Cornus stolonifera* extract produced a highly significant inhibition at 0.01μg/ml, followed by an even greater inhibition at 0.1 μg/ml. This again gradually lessened and eventually produced a highly significant stimulation at 100 μg/ml. The *Solidago canadensis* extract however remained inhibitory throughout the concentration range (figure 4). Even at 0.001 μg/ml a significant inhibition compared to the control was achieved (t test, $P<0.05$). The greatest inhibition occurred at 1 μg/ml and was still highly significant at 10 μg/ml. The least inhibition however again was seen at 100 μg/ml. ANOVA tests determined a significant general dose response across the concentrations of *Rhus hirta* and *Cornus stolonifera* ($P=0.0389$ and $P=0.0003$,respectiviely) while the dose response of *Solidago canadensis* was deemed insignificant ($P=0.2299$).

DISCUSSION

The three plant extracts, originally selected because of their use in treatment of symptoms related to diabetes and subsequently for their high antioxidant capabilities (McCune and Johns, manuscript A), inhibited TNF production in human macrophage. The greatest inhibition came from *Cornus stolonifera* with an average inhibition of 44.68% +/- 11.38 at 0.10 μg/ml and 36.73% +/- 0.99 at 0.01 μg/ml. *Solidago canadensis* reached 30.89% +/- 7.63 inhibition at 1.0 μg/ml and *Rhus hirta* 29.13% +/- 5.94 at 0.01μg/ml.

The biphasic results for *Rhus hirta* and *Cornus stolonifera* may be attributable to a complex mixture of compounds in these extracts. It is possible they contain both inhibitory and stimulatory compounds or that interactions occur among compounds. At lower concentrations (0.01 and 0.1 μg/ml) the inhibitory compound(s) appear to act upon the macrophage TNF production. When the concentrations are increased to 100 μg/ml it

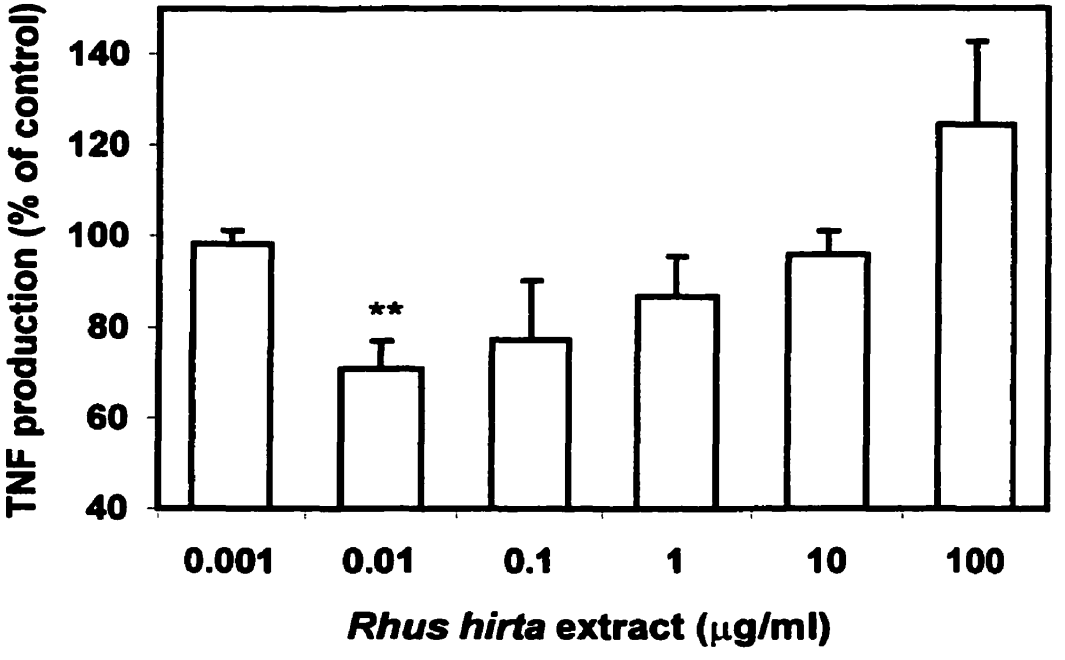

Figure 2. The dose response of *Rhus hirta* extract additions on TNF production in U937 macrophage cells stimulated with LPS. The control, as 100%, is the amount of TNF produced from the macrophage cells stimulated with LPS. The data are expressed as mean +/-standard error (t-test in relation to control,**P<0.01).

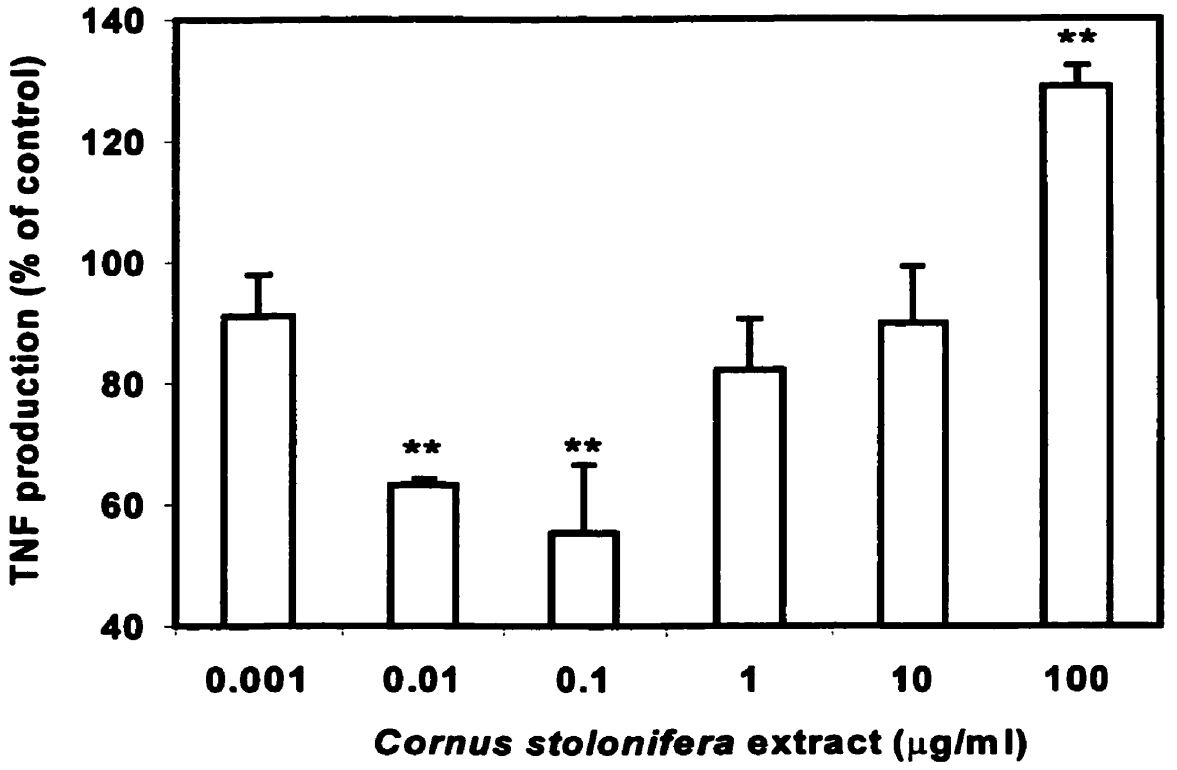

Figure 3. The dose response of *Cornus stolonifera* extract additions upon TNF production in U937 macrophage cells stimulated with LPS. The control, as 100%, is the amount of TNF produced from the macrophage cells stimulated with LPS. Data are expressed as mean +/- standard error (t-test in relation to control,**P<0.01).

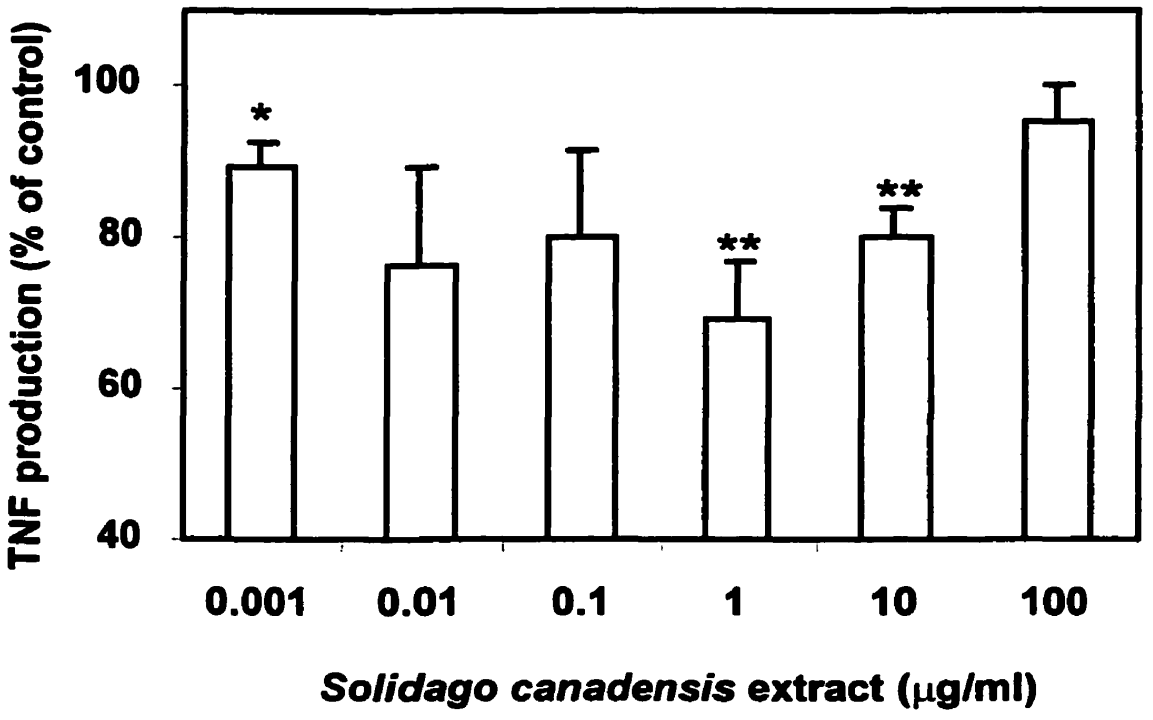

Figure 4. The dose response of *Solidago canadensis* extract additions on TNF production in U937 macrophage cells stimulated with LPS. The control, as 100%, is the amount of TNF produced from the macrophage cells stimulated with LPS. Data are represented as mean +/- standard error (t-test in relation to control,*P<0.05,**P<0.01).

appears that a stimulatory compound(s) reaches a threshold such that its activity now overpowers the activity of the inhibitory compound(s). Chang, *et al.* (1995) reported a similar biphasic response in an extract of a Chinese medicinal plant (*Evodia rutaecarpa*). In that case lower concentrations produced a stimulation of TNF production.

Solidago canadensis with a less pronounced biphasic effect, inhibited TNF production over the whole concentration range. Perhaps the significant inhibitions found at 0.001, 1.0 and 10.0 μg/ml (t-test, $P<0.05$, 0.01 and 0.01 respectively), could be achieved for 0.01 and 0.1 μg/ml with a decrease in variability between the experimental replications at the latter concentrations. The slight increase in cell death at 100μg/ml (also seen in *Rhus hirta)* may indicate a decrease in health of the cell culture at the higher concentrations of extract with a possible affect on the results. However, the general pattern of decreased inhibition with increased concentration is also present with *Cornus stolonifera* where no change in cell viability over different extract concentrations was seen.

The significant stimulation of TNF production by green tea may also be caused by a mix of compounds present in the crude extract. Green tea extract is known to have effects on the immune system (Yan,1992; Jeng *et al.*,1996) and most of the literature on green tea and cancer emphasizes antioxidant capabilities (Katiyar and Mukhtar,1997). One study on epigallocatechin gallate (EGCG), the most prominent antioxidant polyphenol in tea, revealed an inhibitory action on TNF production in a mouse macrophage cell line (Yang, *et al*, 1998). However, Sakagami *et al.*(1995) suggests a stimulation of TNF synthesis in human blood mononuclear cells. Both papers found a discrepancy between different cell lines.

We are not aware of any reports on the affect of a crude extract of green tea on TNF production. The stimulation of TNF reported here might be important in green tea's action on cancer and the immune system. Our results show that at 10μg/ml green tea extract has a stimulatory effect on TNF production of almost 20% in human macrophage

cells. It's possible a more in depth study on dose response with green tea extract would result in a biphasic response, with other concentrations causing the inhibition as seen in Yang *et al.* (1998). Ideally results should be compared to TNF levels in tea drinking human diabetics.

Black tea stimulated TNF production less than green tea. Perhaps due to the destruction or oxidation of some of its constituents during the fermentation process in black tea. Coffee also appeared to have some stimulatory properties. Since the TNF is produced via the immune system and considering that coffee has been suggested to modify the immune system (Melamed *et al.*, 1990), it is not surprising that inhibition of TNF was not seen in these experiments. Coffee has also been reported to increase blood glucose levels (Jenkelson *et al.*, 1967). The results presented here suggest some traditional remedies, often drunk as teas or tonics, may be more beneficial to a diabetic (in relation to TNF inhibition) than the modern beverage (i.e. tea and coffee).

It was reported by Eugui *et al.*(1994) that ascorbic acid and α-tocopherol in the range of 50-200μM insignificantly inhibited cytokines (including TNF). In our experiments ascorbic acid was tested at 56.7μM and Trolox® (an analog of tocopherol) at 39.9μM. Although ascorbic acid showed no inhibition it did produce a significant stimulation of TNF production. Jeng *et al.*(1996) also observed vitamin C to increase TNF production in human mononuclear cells. Perhaps this stimulation is related to vitamin C's purported enhancement of immune function or its inhibition of cancer growth (Kao *et al.*, 1993). Our results, showing no significant inhibition (or stimulation), for the Trolox® form of tocopherol are consistent with those of Eugui *et al.* (1994). Vitamin E's effect on aspects of prediabetes (Salonen *et al*, 1995), insulin action (Paolisso *et al.*,1993) and certain complications of diabetes including cardiovascular disease (Stampfer and Rimm 1995), appears to be unrelated to TNF at the levels tested. As predicted lettuce, a negative control, also had no significant activity on TNF production, thus suggesting our results are not linked to extract preparation.

It is likely the antioxidants in our extracts inhibited the production of TNF through the scavenging of reactive oxygen intermediates required for the activation of the factors (ie NF-kappaB) needed for transcription of the cytokine genes (Eugui *et al.*, 1994). Since most antioxidants in plants are phenolic in nature (Pratt, 1992), it is likely that these type of compounds in the extracts are influencing the TNF production. Tannins, which are also often antioxidant components of plant extracts, are known to occur in *Rhus hirta.* Tannins can inhibit enzyme activity by binding proteins. However various concentrations of pure tannins evaluated in the ELISA kit used in these experiments did not influence the TNF concentration determinations of the kit itself. Therefore any activity recorded are the result of the extracts upon the cell cultures rather than the tannin on the kit assay.

It appears that lower concentrations of these three medicinal plant extracts produce inhibition while higher concentrations may produce stimulation. It is possible that different compounds within these crude extracts act in different ways, at different threshold levels, to produce either a stimulation or inhibition of TNF. This could be similar to vitamin C's known ability to be an oxidant or antioxidant depending on its concentration and presence of certain metals or other antioxidants. In addition to the biphasic TNF effect seen in *Evodia rutaecarpa* extracts (Chang *et al.*,1995) it is worth noting that there are known plant compounds in other plants than can cause immunostimulatory effects such as the panaxans of ginseng (Gao *et al.*,1996). Considering that these plant species are usually ingested via a tea, the levels ingested/cup are probably minute. It remains to be seen in animal models whether the levels of extract ingested and absorbed in various teas cause the same results on TNF production as witnessed in this *in vitro* model.

CONCLUSIONS

All three of the plants used in traditional remedies for diabetes and its complications can influence TNF production in macrophage and therefore likely insulin resistance. Further studies in human diabetics or diabetic *in vivo* models could determine appropriate doses

and direct influence on insulin resistance. *Cornus stolonifera* with its high inhibition of about 45% and 37% at 0.01 and 0.1 μg/ml respectively, dose curve, and low toxicity in this assay stands out as a candidate for further testing. *Rhus hirta*'s strong inhibition by extracts at 0.01 μg/ml, low toxicity at low doses and long history of traditional use (Erichsen-Brown, 1979) also make it a promising candidate. The favorable inhibition of *Solidago canadensis* in comparison to the other extracts at 10 μg/ml (figure 1), as well as its consistent inhibition in the dose response experiments, suggests that this extract may lead to inhibition in diabetic animal models.

The complex mix of antioxidants in plants used for foods, plant medicines or beverages such as tea should be considered important in a role against diabetes and its complications. Moreover, in our search for new antidiabetic drugs, instead of concentrating on hypoglycemic activity we should also be looking at effects on TNF. The antioxidants ingested as part of a normal lifestyle may, through effects on TNF, be important in decreasing insulin resistance and the oxidative stress associated with the incidence of diabetic complications. Such antioxidant influences present in the traditional lifestyles of Indigenous Peoples therefore help to account for a historically low incidence of diabetes.

FINAL CONCLUSION AND SUMMARY

This is the first report on the antioxidant activity of medicinal plants related to diabetes. Although scientific validation has been undertaken for many of the world's traditional plant remedies for diabetes through the action of lowering blood glucose as shown by Marles and Farnsworth (1995), this work is the first to augment their conclusions by suggesting antioxidant action as another benefit of these traditional remedies. Given the reported importance of oxidative stress to the complications of diabetes (Baynes, 1991; Giugliano *et al.*, 1995; Van Dam and Bravenboer, 1997; Thompson and Godin, 1995) these results suggest the importance of medicinal plants in ways other than hypoglycemic activity to the traditional lifestyle of populations prone to diabetes.

As stated in the *Contributions to Knowledge* section, the work of this thesis has generated the first list of potential antidiabetic plants within Canada. In addition, the antioxidant activities generated for each of these species contributes to the knowledge of each of these species. The general hypothesis of this thesis, that antioxidants in the form of antioxidant activity are found in abundance in these medicinal plants, can be accepted. The vast majority (89%) of the species had free radical scavenging activity significantly greater than the market produce, 14% or more had free radical scavenging activity similar to the antioxidant vitamins E and C and the majority (63% and 97%, respectively) had superoxide and peroxyl radical scavenging activities similar to vitamin C. In comparison to the highly publicized antioxidant activity of green tea, eight species had similar free radical activity, 3 similar superoxide scavenging and 21 similar peroxyl scavenging abilities. Considering that a large percentage of these species are also used for food or beverage (Arnason *et al.*, 1981; Kuhnlein and Turner,1991) it follows that these species provide antioxidant benefits to the traditional lifestyle.

In addition to validating the antioxidant capabilities of traditionally used plants, the traditional gathering and preparation practices were found to favor antioxidants. The parts selected for treatment gave higher activity than predicted from the analysis of many parts from a group of species. Fruit gave the greatest antioxidant activity followed by bark, leaves

and the least activity was found in the roots of the selected plant parts used medicinally. The antioxidant activities of these species are most likely in the form of phenolics (Pratt, 1992). Although the exact chemical nature of these extracts are not known, it is known that fruit can have phenolics in the form of colorful attractants as well as antioxidants in their seeds to maintain dormancy (Salisbury and Ross, 1992; Ramarathnam et al., 1989). Other antioxidants such as tannins and feeding deterrent secondary metabolites are often found in barks (Hagerman and Butler, 1991; Doufour and Wilson, 1994) and leaves can accumulate antioxidant flavonoids and terpenoids in response to certain stresses (Anderson and Kasperbauer, 1971; Strack, 1997; Stafford, 1990).

Collection practices were found to favor antioxidant activity in those plants that are perennial, trees or shrubs, and are found in woodland or rocky areas rather than boggy terrain. These results coincide with those of Moerman (1994) who found perennials were favored over annuals in general medicinal plant collection, but not with his finding that herbs were the most often collected as compared to trees and shrubs. This could be a factor of the selection of antioxidant action in this set of plant treatments or the concentration of plant type within this selected growing region as compared to all of North America. Moerman did find that trees listed in his database were used more often medicinally than could be predicted by the plant families it contains.

The conclusions based on the results of these collection practices could direct the discovery of other plants with high antioxidant activities. Ethnobotanical records are critical considering the activities of individual parts in general were considerably different than those used medicinally. The secondary metabolites that may account for some of the antioxidant activity of these plant species could be a result of these species being subjected to a longer lifetime of stresses (i.e. perennials, trees, shrubs). Examples of such accumulation include carotenoids and phenolics in response to UV stress and the development of secondary metabolites upon insect damage and competition (McKey, 1979; Polle and Rennenberg, 1993; Larson, 1988). Research into traditional gathering sites and harvesting techniques would give additional information to practices that may enhance the antioxidant capabilities of these medicinal plants.

This thesis also supports ethnobotanical data that documents the use of a plant species for a symptom or multiple symptoms related to diabetes. Analysis of the symptoms used to select the plant species revealed that the greater the number of symptoms a species was used for (i.e. over 5 symptoms), the greater the activity. Cluster analysis determined that a plant species used for a combination of diarrhea and heart disease or a combination of tonic, wounds, urinary, blood, pregnancy and abscesses could be an predictor of high antioxidant activity. It was also determined that certain symptoms including heart disease, diarrhea, urinary issues, circulation and blood disorders were more likely to have higher antioxidant activity while symptoms associated with infections or general medicines had lower activities. These high antioxidant activities correspond to the literature dealing with oxidative stress in coronary heart disease, lipid peroxidation, microangiopathy and macroangiopathy (Stephens *et al.*, 1996; Stampfer and Rimm, 1995; Cotter *et al.*, 1995; Van Dam and Bravenboer, 1997). Pharmacological treatments of infections by oxidants in the form of hydrogen peroxide solutions or lotions also give some validity to the few symptoms associated with low antioxidant activity.

Presented here was the first study of potential antidiabetic plants in an *in vitro* TNF model, hopefully leading the way for similar studies. Knowing that TNF is a factor in insulin resistance (Hotamisligil *et al.*, 1994; Kahn, 1995), and that some antioxidants have been found to inhibit TNF (Eugui *et al.*, 1994; Sato *et al.*, 1997), three species high in antioxidant activity were selected to test for their ability to inhibit the production of TNF. Considering that they did exhibit the ability to inhibit the production of TNF at various concentrations, *Cornus stolonifera*, *Rhus hirta* and *Solidago canadensis* would all make promising candidates for further testing in a diabetic *in vivo* model to determine dose, toxicity and direct mechanistic affects on TNF production.

The results presented here on all 35 of these potential antidiabetic plant species leads the way for mechanistic studies, whether *in vitro* or *in vivo*, on the extract actions not only in relation to TNF inhibition but other specific complications of diabetes such as atherosclerotic disease, retinopathy and neuropathy. Studies on antioxidants, in particular

vitamin E, are becoming increasingly prevalent in conjunction with many aspects of diabetes. Low levels of vitamin E in plasma can be a risk factor for the development of diabetes (Salonen *et al.*, 1995), vitamin E may also improve glucose disposal (Paolisso *et al.*, 1993) and has been found to improve many of the complications of diabetes (Reaven *et al.*, 1995; Keegan *et al.*, 1995; Rosen *et al.*, 1995; Cotter *et al.*, 1995. As well, antioxidants in green tea have been suggested to be of benefit in the reduction of cardiovascular disease (Tijburg *et al.*, 1997) and to have shown some antidiabetic activity (Matsumoto *et al.*, 1993). Perhaps these medicinal plants may likewise warrant some additional testing in these areas.

Although this thesis has emphasized the antioxidant capabilities of these plants in relation to diabetes, their identification as potential antidiabetic treatments may warrant further studies into possible hypoglycemic action. There may be components of these extracts that have antidiabetic benefit other than antioxidant action. An HPLC analysis of these crude extracts could determine the components of the crude extracts responsible for the antioxidant activity, whether known or unknown. Preliminary tests could identify the levels of typical antioxidants such as tannins, ascorbic acid, tocopherols or flavonoids. More detailed analysis could also determine if there is some level of interaction between the components that adds to the level of antioxidant activity.

The plant species identified here as highest in antioxidant activity would be the most interesting in further testing as antioxidant supplements. *Rhus hirta* fruit, the top in all three of the antioxidant assays, makes a particularly interesting candidate. It has a long historic use as a beverage or tonic, which suggests palatability and possibly low toxicity. It also has a close relation to *Rhus glabra*, which already has been listed in the US Pharmacopoeia (from 1820-1920), and *Rhus aromatica* that is used in the Mediterranean as a spice. *Rhus hirta* is already known to contain gallic and malic acids, tannin and vitamin A (Kuhnlein and Turner, 1991; Turner and Szczawinski, 1979). It is most likely a combination of these, as well as unknown components, that contribute to its antioxidant activity. In addition there is reported hypoglycemic action of *Rhus hirta* fruit (Fortier, 1949).

A detailed literature review in relation to dosage rates, along with interviews of traditional herbalists, could provide a better picture of usual dosage rates of these plant species, and combined with food intakes a more complete picture of antioxidant contributions in the traditional lifestyle. A random sample of water extracts and their antioxidant activity was presented in manuscript B, however further studies could be done on similar water extracts of all 35 plant species (or water extracts specifically following a traditional herbalist preparation) in order to compare the results of those to methanol extracts in relation to antioxidant activity and HPLC analysis of components. This would give a clearer picture of just what could have been ingested in relation to these plant treatments. Toxicity studies of each of these species could also provide needed information, in combination with dosage rates, to determine safe levels of intake.

In analyzing traditional medicinal plant species in an analytical laboratory setting, we were able to scientifically validate the use and selection of these medicinal species as a possible means of contributing to the overall health of Indigenous Peoples of the boreal forest. This was achieved by focussing on an underlying all-encompassing medical condition (oxidative stress) that is also be important in the plant community (i.e. oxidative or environmental stress). This has led to a new way of evaluating traditional knowledge. It's an analytical technique that could be used worldwide that incorporates knowledge of the local flora in the selection of plants for the treatment of symptoms or multiple symptoms. The results suggest the traditional herbalist selected these species in a non-random fashion, possibly based on characteristics associated with antioxidants. These characteristics could encompass taste (e.g., astringent, which are often associated with antioxidants), particular plant tissues, structures or habitats (and therefore environmental stresses), as well as knowledge gained over time through the alleviation of symptoms. As we increase our scientific knowledge base in regard to phytochemistry and medical conditions we acquire a better understanding of the past uses of plants and find ourselves rediscovering the traditional lifestyle and its benefits.

EPILOGUE

The results presented on some of these medicinal plants lead to exciting speculation. In particular *Rhus hirta* shows exceptional promise as an antioxidant supplement. With antioxidant activities equal or greater than vitamin C and a long history of use, several additional reports were produced regarding this medicinal species. These reports are presented in appendix 2.

Figure 1. *Rhus hirta*

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APPENDICES

Preliminary Analysis of Extraction Technique

Description of Analysis

Considering the high number of plant samples to be evaluated in various assays in this thesis work, it was imperative to determine the most efficient extraction technique. A general discussion on extraction method is available in manuscript B, however some preliminary experiments were necessary to determine the best extraction method using alcohol. Alcohol was the extractant of choice to insure the least amount of enzyme degradation of the sample (Harborne, 1973) and greatest extraction of compounds of various solubilities.

There are numerous methods employed in the literature for extraction of plant material. Typically extractions in the laboratory of Johns, similar to that of Ducki *et al.* 1996, had been done using 1 part dried ground sample to 7 parts methanol for an extraction period of 5-7days at room temperature (Lindhorst, 1998). In order to compare the antioxidant activity of different extraction times various procedures were carried out on a mixture of dried plant material and methanol. Using green tea, black tea, coffee, onion and garlic comparisons were done on 20g of material in 200 mL of methanol. The flasks were placed on a shaker at room temperature over a period of days followed by the removal of extractant via vacuum filtration, rotary evaporation and lyophilization. Four different extraction procedures included: 1) the combined daily extractions for 5 days on the same sample, 2) the combined extractions on days 7 and 12 after procedure #1, 3) one extraction after 5 days and 4) one extraction on day 12 after procedure #3.

Some of the results of these procedures on the free radical activities of the extracts are illustrated in figure 1. Following this exercise it was determined that there was no great difference in antioxidant activity in extracting daily compared to once after 5 days. The yields for green tea were 37% (w/w) for the combined above procedures #1 and #2 and 32% (w/w) for procedures #3 plus #4. Since solubility problems occurred with the onion and garlic extracts in ethanol for the DPPH free radical assay (discussed below), analysis of this assay using methanol instead of ethanol was conducted (data not shown). No appreciable

difference in results was noted between the alcohols. Considering the plant extractions would be carried out in methanol, and methanol is considered a better solvent than ethanol (Windholz, 1983), this substitution was made in the DPPH free radical assay of Cotelle *et al.* (1996) used throughout this thesis.

In order to decrease the time spent on extracting, a Soxtec extraction method was also evaluated. Similar procedures involving refluxes have been used by Schmeda-Hirschmen *et al.*, 1987 and Yoshikawa, 1995. Considering this machine is based on the method of using a Soxhlet apparatus, this original method was compared to the quicker Soxtec machine. Using 20g of green tea the Soxhlet apparatus approach took a full day while the Soxtec machine with 3 refluxes took 3 hours with the ability to do two at a time. Another Soxtec machine was tried as well that had the ability to do more samples at once, however the machine was in greater demand and the samples had to be of smaller size.

In comparing the various methods for differing antioxidant activity (see figure 2), it was determined that the quicker Soxtec extraction procedure provided similar results to the slower methods. The yields of green tea were 41% (w/w) for both the Soxhlet apparatus approach and the Soxtec machine. In this manner extraction procedure was reduced from a minimum of 5 days to a possible daily extraction of 4 samples (2 samples/ machine/ 3hours coupled with rotary evaporation, etc.). Concern over the high temperatures used in the Soxtec were alleviated with a comparison of standard antioxidants and flavonoids (ascorbic acid, tannic acid, quercetin, rutin, cinnamic acid, α-tocopherol and epicatechin). There were no significant differences in their activity after the Soxtec procedure as compared to fresh solutions in the DPPH free radical assay (data not shown).

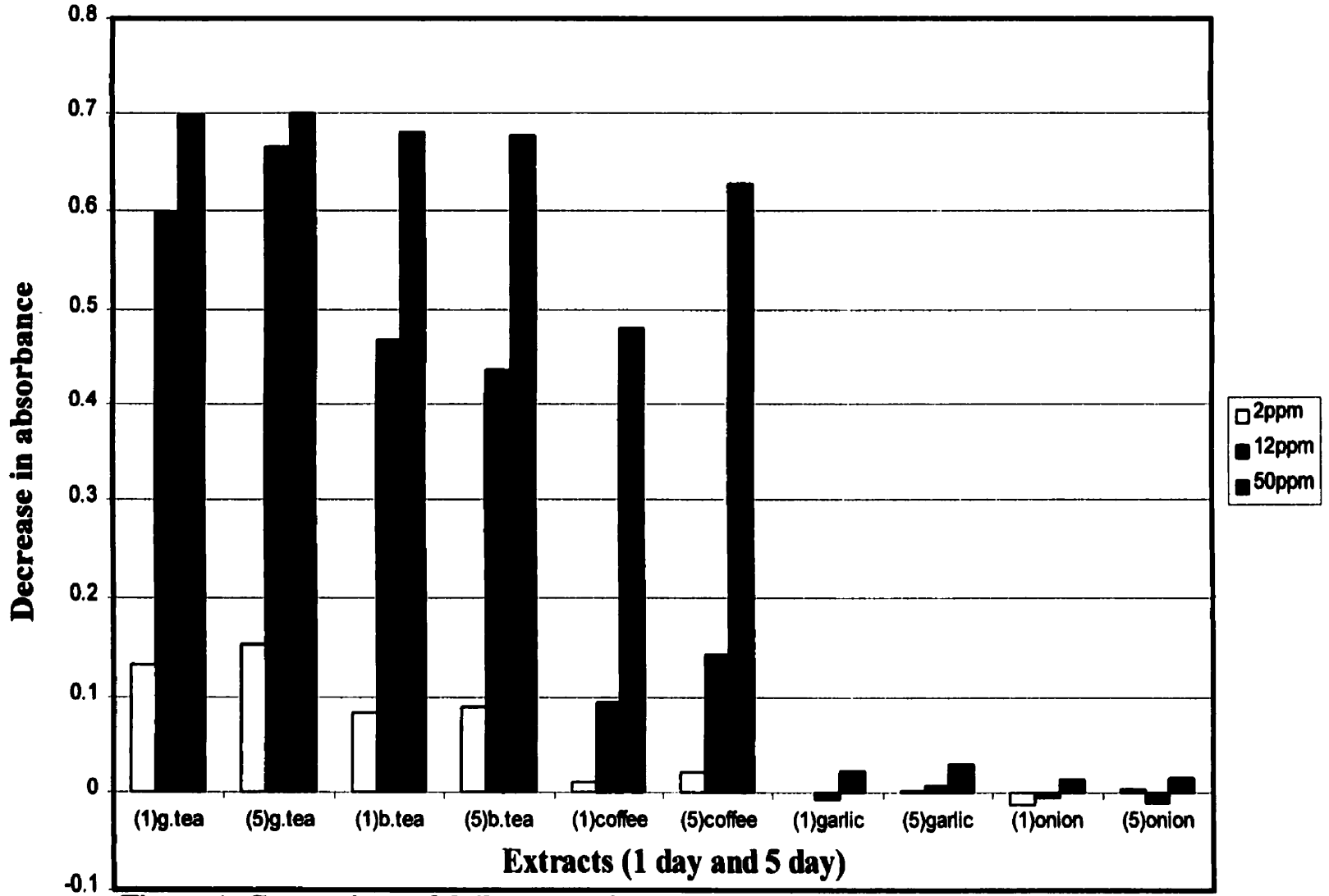

Figure 1. Comparison of daily extractions versus a single extraction over 5 days

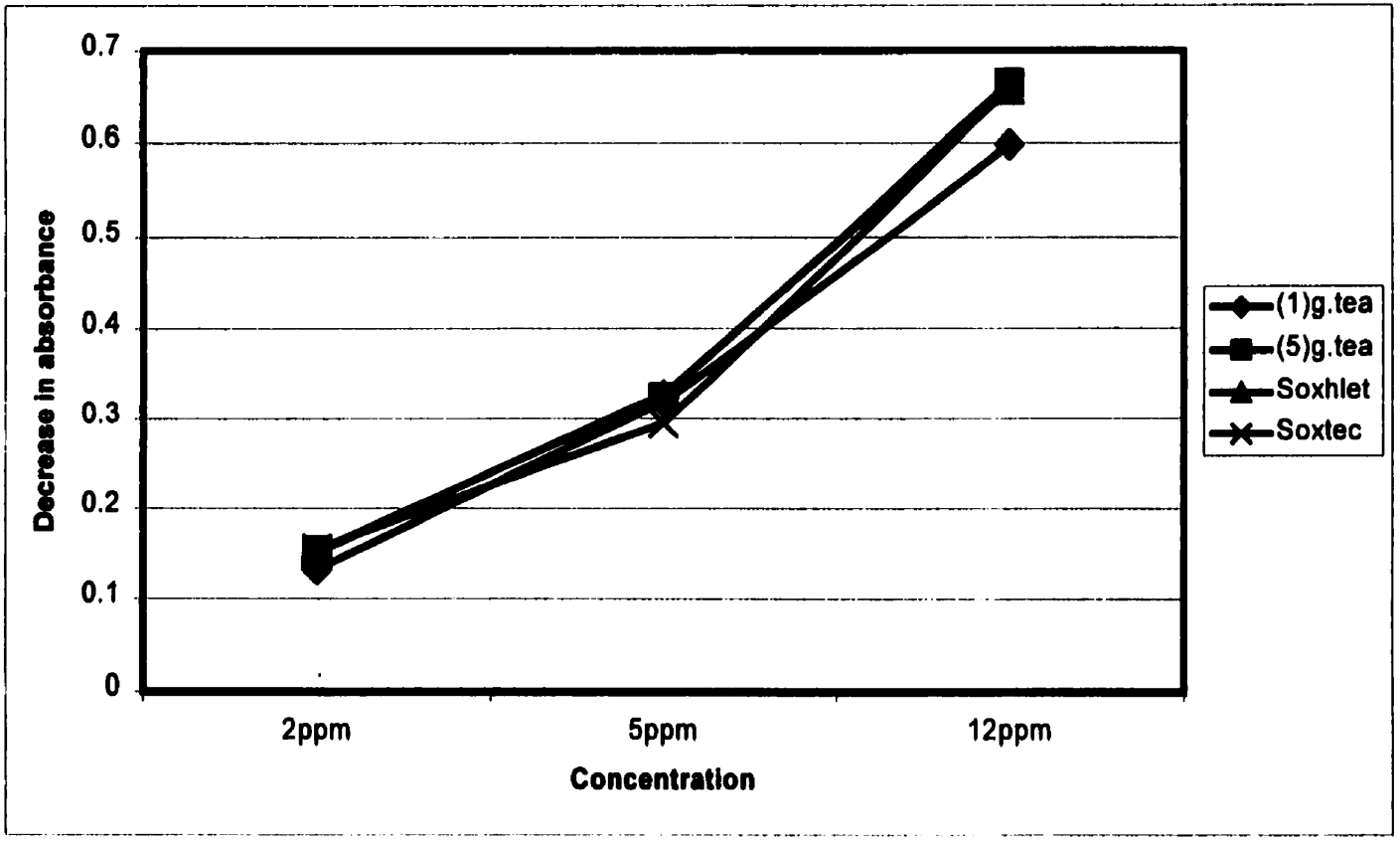

Figure 2. Comparison of Soxtec to other methods of extraction on green tea

LETTER TO CINE GOVERNING BOARD

Rhus hirta research

by Letitia McCune and Tim Johns

This work was carried out in partial fulfillment of thesis work in the Department of Plant Science. The work itself is largely based in the laboratory. The 35 different plant species selected for laboratory work were determined **solely** by reviewing published literature. *Rhus hirta* was one of these species. Plant collections were largely done in the Montreal area. There was **no** collection of data, information or plants from Indigenous communities, Elders, Spokesmen, etc.

The premise of the thesis was largely based on the following: 1)It is known that there is an increase in diabetes among Indigenous People when the lifestyle is changed from the traditional hunter gatherer to the more sedentary market consumer. 2) The effect of the reduction of traditional medicines as part of that traditional lifestyle has been ignored .3) Rather than focus on the "medicinal" aspects of these traditional medicines or plants we focussed on their antioxidant activity. 4) Antioxidant activity is usually studied in relation to vitamins A, C and E amounts in fruits, vegetables and grains consumed as foods or beverages. 5) Recent scientific literature has found that certain levels of these antioxidant vitamins can decrease many of the complications of diabetes (ex. arterial and eye problems) as well as the incidence of diabetes among prediabetics.

In order to show that medicinal plants may also contribute to the antioxidant benefit of the traditional lifestyle, aside from the traditional foods, we needed to select plants to analyze for antioxidant activity. Considering the known benefit to those prone to diabetes we decided to concentrate on those plants used for a combination of symptoms in any way

related to diabetes or its complications (ex. heart problems, leg problems, eyes, fatigue, urinary infections, etc.). Considering the high incidence of diabetes among the Indigenous Peoples located in Canada plants used by these groups were particularly highlighted. This was achieved by using the compilation of literature (dating as far back as 1891) on food and medicine use of plants by Arnason *et al.*(1981). Thirty-five plant species were thus selected and easily collected in the areas near campus.

Three laboratory assays on general antioxidant activity on the dried and ground collected plants have shown high antioxidant activity in many of these plants. Although we don't known the exact cause of this activity, many of these are already known to contain some of the well known antioxidant vitamins A, C and E. This seems to confirm our theory that the medicinal plant consumption in the traditional lifestyle contributes to the low incidence of diabetes attributed to this lifestyle.

Rhus hirta is of particular interest because its use as a tonic and beverage suggests that it can contribute greater amounts of antioxidants to the traditional diet than would be consumed if it was used strictly as a medicine. Its use and acceptance as a dietary supplement is promising considering the long historical use of it and its close relatives as a wild beverage and sour treat (see Erichson-Brown,1979). Close relatives are also widely used as a spice in the Middle-East. The US pharmacopoeia has listed for a number of years the use of *Rhus glabra* (the western equivalent of *Rhus hirta*).

Description of the plant Rhus hirta:

This species is also known as *Rhus typhina*. Common names have included staghorn sumac, lemonade tree, vinegar tree, hairy sumac and velvet sumac. The latin species name *typhina* suggests an appearance similar to cattails (*Typha* spp.) and the more recent use of *hirta* is latin for hairy. The common names staghorn sumac, hairy sumac and velvet sumac refer to the branches being covered in soft hairs similar to deer antlers. The common names vinegar tree and lemonade tree refer to its use for "Indian lemonade" made by mashing the berries with boiling water, straining and adding sugar as needed.

The fruit of this weedy shrub is reddish brown and occurs in thick pyramidal or cone-like clusters of hairy 'berries' or seeds. Fruit is usually collected in early July prior to any rain but sometimes into the fall as well. It is the hairs of this fruit that give the characteristic red color. It grows in clearings, fields and dry ground- often seen along the roadside. It is a weedy species and grows easily in the range just north of Lake Superior and east to Nova Scotia in Canada and in the US in New England south to North Carolina, in the mountain regions of Georgia and as far west as Kentucky, Illinois and Iowa (as described in the Audubon's field guide to wildflowers). It is also known to be grown ornamentally in gardens of Europe (though this may be *Rhus coriaria*-the native species of that area).

Related species

It is placed in the family Anacardiaceae, also known as the cashew family. Economic plants of this species include not only the cashew but the mango, pistachio and varnish tree. Tannins from some of these plants, including Rhus typina, have been used in the past for tanning purposes. *Toxicodendron* is one genus group within this family that include the poison-ivy, poison-oak and poison-sumac.

Unfortunately the common name 'sumac' may create confusion in discussions with those who do not know the difference between the 'poison sumac' (*Toxicodendron vernix*) and *Rhus hirta.* Reassurances can be made that there is no likelyhood of confusing any potential product formed from the fruit considering *Rhus hirta* fruit is an upright dense cluster or cone of red hairy seeds. *Toxicodendron vernix* has glossy white berries in a dangling strand-like formation. In addition the plant's leaves are not toothed like *Rhus hirta*'s nor do they have the characteristic velvet covering of the branches. And of course *Rhus hirta* does not produce the irritating reactions common to *Toxicodendron vernix.* That is part of the reason the *Rhus* species are not placed now in the same genus with those that have been moved to the toxic *Toxicodendron* genus. Any customer confusion can be averted by calling a product from *Rhus hirta* as coming from Indian lemonade, vinegar tree, lemonade tree, etc.

Rhus glabra or *Rhus aromatica* in the Mediterranean also has similar fruit structure. The fruit is often ground and mixed with salt as a spice. In Lebanon, Syria and Egypt a thick syrup is made from the fruit and used as a sour replacement for dishes using the indian date (tamarind).

There is history of the use of smooth sumac (*Rhus glabra*) in the US pharmacopea. *Rhus glabra* forms the same type of fruit as *Rhus hirta* but in more open clusters. As the name implies its bark is smooth and therefore easily distinguished from *Rhus hirta*.

Known medicinal uses of Rhus hirta specifically:
As summarized from Arnason *et al.*,1981 and Moerman,1998.

Algonquin	tonic, antirheumatic, dietary aid, general medicine
Cherokee	antiemetic, burn dressing, gynecological aid, urinary aid
Chippewa	analgesic, gastrointestinal aid
Delaware	diarrhea, venereal aid
Iroquois	dietary aid, frequent urination, warming intestine, improve milk, before birth ,wounds, gynecological aid, reproductive aid
Malecite	purification of blood, cough medicine, febrifuge, tuberculosis remedy
Menominee	cough medicine, dermatological aid, gastrointestinal aid, gynecological aid, hemorrhoid remedy, pulmonary aid, tuberculosis remedy
Meskwaki	anthelmintic
Micmac	sore throat, dietary aid
Mohegan	throat aid
Natchez	dermatologic aid
Ojibwa	hemorrhage, sore throat, mouth sore, stomach pain
Potawatomi	anthelmintic, hemostat, sore throat
Rappahannock	general medicine

In conclusion:

The general thesis work is exciting in its further confirmation of the benefits of the traditional lifestyle in preventing diabetes and its complications. It also serves to help break some of the stereotyped grouping of foods separate from medicines. By showing the antioxidant potential of medicinal plants in the traditional "diet" we lead the way for further studies in consumption patterns and total levels of antioxidants in the traditional lifestyle.

As an offspring of this work the potential of *Rhus hirta* as a dietary supplement was realized when its antioxidant activity, long historic use and relation to world-wide spice use was reviewed.

McCune and Johns

Report of Invention

***Re: Rhus hirta* as a natural antioxidant supplement**

A: Background of invention

Description of the plant Rhus hirta:

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past for tanning purposes. *Toxicodendron* is one genus group within this family that include the poison-ivy, poison-oak and poison-sumac.

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Diabetes use:

Banting, a coinventor of insulin, first scientifically studied *Rhus typhina* leaves as a possible antidiabetic after learning of their use by the Indigenous People- with no success. In 1949 a publication in Laval Medicial by G. Fortier proved an extract of the fruit produced hypoglycemia (lowering of blood sugar) in dogs. The European species, *Rhus coriaria,* has been shown to lower glucose in diabetic rabbits.

Known constituents:

Fortier (1949) reviewed the literature for constituents of the fruit and found it to have 12.2-12.3 parts per hundred of fats, 6-10 parts per hundred of malic acid, 16 parts per hundred of gallotannins and a presence of glyceric acid and anthocyanins.

A unique triterpenoid has recently been described from *Rhus typhina* flowers. The leaves have been analyzed recently for volatile constituents.

B. Technical description:

The two attached paper manuscripts describe the experiments that determined *Rhus hirta*'s antioxidant activity and action against tumor necrosis production. The main points are highlighted below:

- *Rhus hirta* was the best scavenger in all three assays of different types of antioxidant activity as compared to 34 different native species used medicinally. It had a mean activity greater than ascorbic acid (vitamin C) in free radical scavenging, a significantly greater activity than ascorbic acid in superoxide scavenging (and similar to green tea), and a mean peroxyl radical scavenging higher than ascorbic acid.

- *Rhus hirta* produced a significant inhibition (29.13+/-13.0 %) of TNF production in macrophage cells at the concentration of 0.01ug/mL in vitro. TNF is important in producing reactive oxygen species as well as insulin resistance to peripheral cells (an important part of type 2 diabetes).

Fruit over other part of Rhus hirta:

In testing different parts of several species we found *Rhus hirta*'s fruit to be the highest in activity compared to other parts with an activity of 3.73 +/-0.07ppm while the root was

4.77+/-0.60ppm, the stems 6.72+/-0.64ppm and the leaves at 5.44+/-0.21ppm. Even so, these other activities are quite high in and of themselves.

Fractionation:

In addition we fractionated the methanol extract into chloroform, ethyl acetate and water. The free radical antioxidant activities of these fractions were 25.96ppm, 2.45ppm and 9.64ppm respectively (experiment performed three times in duplicate).

Water extracts:

We also tested the antioxidant activity of a water extract or "tea" made from fresh fruit (2 cups fruit + 2 cups filter water, mashed, let sit for one hour, brought to a slight boil, cooled with a cover, frozen and freeze dried). The free radical activity of this was found to be 10.75ppm- not quite as high as the methanol extract. However, previous experiments on the same fruit frozen and used for the methanol extract gave water extract results of 3.90+/-0.12ppm for dried material and 3.94+/-0.24ppm for fresh frozen fruit. These water extracts were performed more scientifically in the laboratory as a combination of infusion and decoction. Twenty grams of powdered sample (if dried) was added to 40 mL of distilled water for 5 minutes. 160 mL was then added followed by gentle mixing for 10 minutes and filtration. The residue was further extracted for 15 minutes with 200mL boiling water before filtration. In the third stage the residue was extracted by simmering for 15 minutes in 200 mL of water and then filtered. The three extracts were pooled before lyophilization.

C. General purpose and commercial applications:

This would serve to be a natural antioxidant, as an herbal supplement, more active than ascorbic acid. Antioxidants are recently very prevalent in the scientific and popular literature regarding anti-cancer and anti-aging in particular in relation to wine, green tea and bioflavonoids.

The herbal supplement market has been estimated at $587,336,112.00 USD over a 52 week period ending May 17,'98. This was up 101% in the mainstream market from the year before. Another source, from the *Journal of the American Medical Association*, suggests the amount could be as high as $5.1 billion. Sales of Echinacea and Goldseal account for $63,553,612 of the $587,336,112 figure.

Considering that antioxidants are important to diabetics (see papers) it should be noted that the diabetic population continues to grow. The WHO has estimated that by the year 2010 diabetes will affect 221 million people worldwide. The American Diabetes Association says 15.7 million or 5.9% of the population of the US has diabetes. It is considered one of the most costly health problems in the US at approximately $92 billion per year.

Antioxidant sales in France were up to 32 million francs in 1998. Supplements labeled antioxidants are mainly comprised of vitamin C supplements sometimes in combinations with Vitamin E or carotenes. Grapeseed and Pycnogenol (pine resin) could also be considered in the antioxidant market, in 1998 the total US dollar sales of these was $11,074,712.

The leading companies:
Attached is a published list of the top companies that may deal in plant-based pharmaceutical products or supplements. Tim Johns has personal connections with Shaman Pharmaceuticals and Forbes.

Potential patent lawyer:
Sechley, Konrad, A *et al.*
Gowling, Strathy and Henderson
Suite 2600, 160 Elgin Street
Ottawa, Ontario K1P 1C3

Determined from a search of www.patents.ibm.com site under plant+extract+diabetes (2 hits= W009852587A1 and W009703676A1).

Preliminary patent search:

Using the ibm site there were no hits for rhus+typhina, rhus+hirta, sumac+staghorn, sumac+antioxidant, sumac+diabetes or rhus. There were 9 under sumac, 214 under plant+extracts and 3 under plant+extract+antioxidant.

Using the ic.gc site there were no hits under *Rhus typhina* or *Rhus hirta*, there was a smoking substitute mix that contained some staghorn sumac and there were 6 hits with extract+plant+diabetes.

D. Advantages and improvements over existing technology:

Natural ingredients as compared to standard antioxidant vitamins
Long historical use as "lemonade",etc.
Greater antioxidant activity than ascorbic acid(vitamin C)
Potential as a drink as well as a supplement