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By

Syed Mubasher Sabir

Antioxidant and hepatoprotective activity of Brazilian plants, containing phenolic compounds

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Syed Mubasher Sabir

Supervisor

Prof. Dr. Joao Batista Texeira da Rocha

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The undersigned examining committee, approved the thesis entitled

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to

Syed Mubashar Sabir

Examiners

Prof. Dr. Joao Batista Texeira da Rocha Orientador(UFSM)

Prof(a). Dr.(a) Maribel Antonello Rubin (UFSM)

Prof(a). Dr (a) Nilda Berenice de Vargas Barbosa

Prof. Dr. Robson Luiz Puntel(UNIPAMPA)

Prof(a) Dr(a). Margareth Athayde(UFSM)

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Organization of the thesis

The results that make up this thesis are presented in the form of written manuscripts and are found under the scientific articles.

The sections **Material and Method, Results, Discussion of results, and References** are found in the articles itself and present an integral part of this study.

The item, General comment is for the purpose of brief and short interpretation on scientific articles presented

The references refer only to citations that appear in the items, introduction and general comments on results.

Index

1. S.M., Sabir, J.B.T., Rocha 2008. Water-extractable phytochemicals from *Phyllanthus niruri* exhibit distinct *in vitro* antioxidant and *in vivo* hepatoprotective activity against paracetamol-induced liver damage in mice Food Chemistry 111, 845-851.

2. S.M., Sabir, J.B.T., Rocha 2008. Antioxidant and hepatoprotective activity of aqueous extract of *Solanum fastigiatum* (false “Jurubeba”)

against paracetamol-induced liver damage in mice. Journal of ethnopharmacology 120, 226-232.

3. S.M., Sabir et al. 2010. Therapeutic implications of *Baccharis trimera* against oxidative stress, *in vivo* paracetamol intoxication in mice and HPLC quantification of the phenolic compounds. Planta Medica (submitted).

4. S.M., Sabir et al., 2010. *In vitro* antioxidant and genotoxic evaluations of ethanolic extract of *Solidago microglossa* containing phenolic compounds. Mutation research (submitted).

Resumo

Plantas da flora Brasileira foram escolhidas e estudadas por sua atividade antioxidante tanto *in vivo* como *in vitro* e também por sua atividade hepatoprotetora *in vivo*, especialmente relacionadas a compostos fenólicos que possuem inúmeros benefícios para a saúde. Essas plantas incluem *Phyllanthus niruri*, *Solanum fastigiatum*, *Solidago microglossa* e *Baccharis trimera*. O extrato aquoso destas folhas mostraram inibição contra a produção de espécies reativas ao ácido tiobarbitúrico (TBARS), induzidas por diferentes pro-oxidantes (10 μM $FeSO_4$ e 5 μM de nitruprussiato de sódio) em homogeneizado de fígado e cérebro de ratos.Esses extratos também diminuiram a formação de TBARS em fosfolipídios extraídos de gema de ovo. No ensaio para peroxidação lipídica a ordem de atividade antioxidante foi *Solidago microglossa> Phyllanthus niruri > Solanum fastigiatum > Baccharis trimera*. A atividade detoxificante de radicais livres dos extratos foi determinada pela quelação do radical 2,2-difenil-1-picrilhydrazil (DDPH). Os valores de IC_{50} para *Solidago microglossa* (3.8 ± 0.1 μg/ml), *Phyllanthus niruri* (43.4 ± 1.45 μg/ml), *Solanum fastigiatum* (68.96 ± 1.25 μg/ml) e *Baccharis trimera* (415.2 ± 15.2 μg/ml) indicam que a ordem de atividade antioxidante é *Solidago microglossa> Phyllanthus niruri > Solanum fastigiatum > Baccharis trimera*. Também foi demonstrada a atividade hepatoprotetora dos extratos contra o dano hepático induzido por paracetamol *in vivo*, evidenciado pela diminuição nos níveis séricos da glutamato oxaloacetato transaminase (GOT) e glutamato piruvato transaminase (GPT), decréscimo na atividade da catalase no fígado do grupo tratado em comparação com o controle. O extrato aquoso mostrou também significativa ($p<0,05$) atividade hepatoprotetora a qual foi evidenciada por verificação de atividade enzimática e restauração dos níveis de TBARS, além disso, restauração a valores normais de forma dose dependente dos níveis de tiol não-proteico e dos níveis de ácido ascórbico. Estudos sobre a toxicidade aguda da *Solanum fastigiatum* revelaram que o valor da LD_{50} para o extrato são maiores que 4 g/Kg de peso corporal de camundongos enquanto que a genotoxicidade da *Solidago microglossa* foi avaliada usando o teste do cometa. Os resultados obtidos no teste do cometa revelaram que nesta condição experimental o extrato não foi genotóxico para linfócitos humanos sob concentrações relativamente altas de 0.5 mg/ml. O conteúdo fenólico foi determinado usando o teste Folin Ciocalteu. A *Solidago microglossa* apresentou o maio teor fenólico enquanto que a *Baccharis trimera* apresentou a menor quantidade. Análises fitoquímicas dessas plantas foram realizadas através de TLC/HPLC. As análises TLC revelaram a presença de rutina no extrato aquoso de *Phyllanthus niruri*, enquanto que na *Solanum fastigiatum* foi observado à presença de rutina e quercetina. O perfil fenólico da *Solidago microglossa* e da *Baccharis trimera* foi determinado por cromatografia líquida de alto desempenho. Baseado nos dados do HPLC foi identificado: quercetina

(254.88 ± 5.1 µg/ml), rutina (342.2 ± 7.1 µg/ml) e ácido gálico (2320.8 ± 13.2 µg/ml) no extrato etanólico da *Solidago microglossa.* Além disso, analises no HPLC revelaram a presença de ácido gálico (76.8 ± 7.2 µg/ml) como o principal constituinte, enquanto que a rutina (20.1 ± 2.5 µg/ml) e quercetina (10.1 ± 1.4 µg/ml) apresentaram uma menor contribuição no extrato aquoso de *Baccharis trimera* . Concluindo, o extrato cru dessas plantas apresentou alta atividade antioxidante e detoxificadora de radicais livres as quais estão relacionadas à presença de compostos fenólicos. Esses resultados implicam no uso dos extratos como agentes terapêuticos na prevenção de doenças relacionadas ao estresse oxidativo.

Abstract

Plants from Brazilian flora were chosen and studied for their *in vitro* and *in vivo* antioxidant and *in vivo* hepatoprotetive activity, especially in relation to phenolic compounds which have several health benefits. These plants include *Phyllanthus niruri*, *Solanum fastigiatum*, *Solidago microglossa* and *Baccharis trimera*. The aqueous extracts of leaves showed inhibition against thiobarbituric acid reactive species (TBARS), induced by different pro-oxidants (10 μM $FeSO_4$ and 5 μM sodium nitroprusside) in rat liver and brain homogenates. The extracts also lowered the formation of TBARS in phospholipid extracted from egg yolk. On the lipid peroxidation assay the order of antioxidant activity was *Solidago microglossa>Phyllanthus niruri> Solanum fastigiatum> Baccharis trimera.* The free radical scavenging activities of the extract were determined by the quenching of a stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The IC_{50} value of *Solidago microglossa* (3.8± 0.1 μg/ml), *Phyllanthus niruri* (43.4 ± 1.45 μg/ml), *Solanum fastigiatum* (68.96±1.25 μg/ml) and *Baccharis trimera* (415.2±15.2 μg/ml) indicates that the order of antioxidant activity is *Solidago microglossa* > *Phyllantus niruri* > *Solanum fastigiatum* > *Baccharis trimera.* The hepatoprotective activity of the extracts were also demonstrated *in vivo* against paracetamol-induced liver damage, as evidenced by the decrease in serum glutamate oxaloacetate transaminase (GOT), and glutamate pyruvate transaminase (GPT), and decreased catalase activity in the liver in treatment groups, compared to the control. The aqueous extract also showed significant ($p<0.05$) hepatoprotective activity that was evident by enzymatic examination and

brought back the altered levels of TBARS, non-protein thiol and ascorbic acid to near the normal levels in a dose dependent manner. Acute toxicity studies of *Solanum fastigiatum* revealed that the LD_{50} value of the extract is more than the dose 4 g/kg body weight of mice whereas, the genotoxicity of *Solidago microglossa* was tested using comet assay. The results obtained on the comet assay revealed that under the experiment used the extract was not genotoxic to human lymphocytes at a relatively high concentration of 0.5 mg/ml. The content of phenolics was determined using Folin Ciocalteu's assay. The phenolic content was found to be the highest in *Solidago microglossa* and the lowest in *Baccharis trimera*. Phytochemical analysis of these plants was carried out on the basis of TLC/HPLC. The TLC analysis showed the presence of rutin in aqueous extract of *Phyllanthus niruri*, while, *Solanum fastigiatum* showed the presence of rutin and quercetin. The Phenolic profile of *Solidago microglossa* and *Baccharis trimera* was determined by using High performance liquid chromatography. On the basis of the HPLC data, quercetin (254.88±5.1 µg/ml), rutin (342.2±7.1 µg/ml) and gallic acid (2320.8±13.2 µg/ml) have been identified and quantified in the ethanolic extract of *Solidago microglossa*. Whereas, HPLC analysis revealed the presence of gallic acid (76.8±7.2 µg/ml) as a major compound while, rutin (20.1±2.5 µg/ml) and quercetin (10.1±1.4 µg/ml) relatively showed minor contribution in the aqueous extract of *Baccharis trimera*. In conclusion, the crude extract of these plants showed high antioxidant and free radical scavenging activities which are related to the presence of phenolic compounds. These

results may have implications in the use of the extracts as a therapeutic agent in the prevention of oxidative stress related diseases.

1. Introduction

1.1. Free radicals

Free radicals are highly reactive molecules or chemical species capable of independent existence. Generation of highly reactive oxygen species (ROS) is an integral feature of normal cellular function like mitochondrial respiratory chain, phagocytosis, arachidonic acid metabolism, ovulation, and fertilization. Their production however, multiplies several folds during pathological conditions. The release of oxygen free radicals has also been reported during the recovery phases from many pathological noxious stimuli to the cerebral tissues [1]. Oxygen, because of its bi-radical nature, readily accepts unpaired electrons to give rise to a series of partially reduced species collectively known as (ROS) including, superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl (HO), peroxyl (ROO), alkoxy (RO), and nitric oxide (NO), until it is itself completely reduced to water. Most of the superoxide radicals are formed in the mitochondrial and microsomal electron transport chain. Except for cytochrome oxidase, which retains the partially reduced oxygen intermediates bound to its active site, all other elements in the mitochondrial respiratory chain, e.g., ubiquinone, etc., transfer the electron directly to oxygen and do not retain the partially reduced oxygen intermediates in their active sites [2]. On the internal mitochondrial membrane, the superoxide anion may also be generated by auto-oxidation of semiquinones. The majority of superoxide radicals generated by mitochondrial electron transport chain are enzymatically dismutated to H_2O_2. The hydroxyl and alkoxy free radicals are very reactive species and rapidly attack the macromolecules in cells [3].

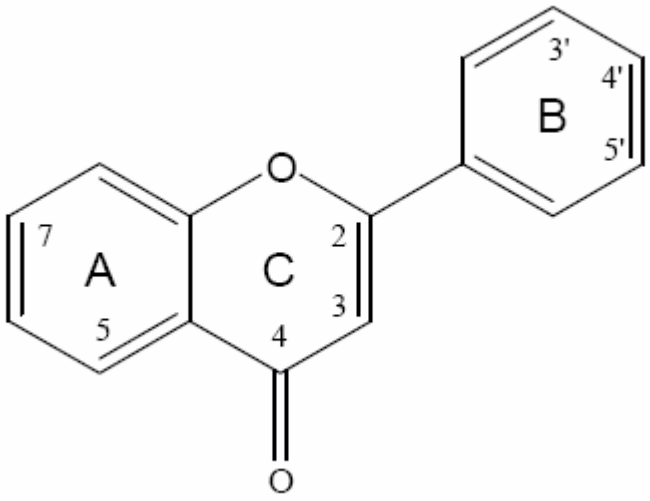
Damage due to free radicals caused by ROS leads to several damaging effects as they can attack lipids, proteins/enzymes, carbohydrates, and DNA in cells and tissues. They induce undesirable oxidation, causing membrane damage, protein modification, DNA damage, and cell death induced by DNA fragmentation and lipid peroxidation. This oxidative damage/stress, associated with ROS is believed to be involved not only in the toxicity of xenobiotics but also in the pathophysiological role in aging of skin and several diseases like heart disease (atherosclerosis), cataract, cognitive dysfunction, cancer (neoplastic diseases), diabetic retinopathy, critical illness such as sepsis and adult/acute respiratory distress syndrome, shock, chronic inflammatory diseases of the gastrointestinal tract, organ dysfunction, disseminated intravascular coagulation, deep injuries, respiratory burst inactivation of the phagocytic cells of immune system, production of nitric oxide by the vascular endotheliums, vascular damage caused by ischaemia reperfusion known as ischaemia/reperfusion injury and, release of iron and copper ions from metalloprotein [4]. Iron changes have been detected in multiple sclerosis, spastic paraplegia, and amyotrophic lateral sclerosis, which reinforces the belief that iron accumulation is a secondary change associated with neuro degeneration in these diseases, although it could also be related to gliosis (glia might produce free radicals) in the diseased area, or the changes in the integrity of the blood brain barrier caused by altered vascularisation of tissue or by inflammatory events [8].

1.2. Antioxidative activity

An antioxidant is a substance that when present at a concentration low compared to that of an oxidisable substrate, significantly delays or prevents oxidation of that substrate [5]. Even though plant phenols are not always treated as real antioxidants in the literature, many *in vitro* studies have demonstrated the antioxidant potential of phenols as direct aqueous phase radical scavengers and as agents capable of enhancing the resistance to oxidation of low density lipoproteins implicated in the pathogenesis of coronary heart disease [6]. It is admitted that a part of the antioxidant capacity of many fruits and berries is derived from flavonoids [7, 8] and, in fact, all the major polyphenolic constituents of food show greater efficacy in these systems as antioxidants on a molar basis than the antioxidant nutrients vitamin C, vitamin E, and β-carotene. Differences between the antioxidant potential of selected compounds can be measured using many different techniques. Because most phytochemicals are multifunctional, a reliable antioxidant protocol requires the measurement of more than one property relevant to either foods or biological systems [9].

Figure 1 Numbering of the flavone nucleus. Certain structure-antioxidant activity relationships of flavonoids can be derived on the basis of the literature.

Comparison between quercetin, catechin and cyanidin demonstrates the



importance of unsaturation in the C ring (**Figure 1**), allowing electron localization across the molecule for stabilization of the aryloxyl radical. The 3-OH group attached to the 2,3-double bond, and its location adjacent to the 4-carbonyl in the C ring, are required for the maximum effectiveness of radical scavenging [10]. The weaker antioxidative potential of catechins can be enhanced to the stage of quercetin by incorporation of the 2,3-double bond and 4-oxo function, by ester linkage via the 3-OH group to gallic acid, and incorporation of an additional 5'-OH group in the B ring [11,12,13]. This results in (-)-epigallocatechin-3-*O*-gallate being one of the most efficient scavengers of the superoxide radical [14]. A mono phenolic ring is not an effective hydrogen donor. Thus the antioxidant activity is at its maximum when the B ring is substituted by two hydroxyl groups in the *ortho*-diphenolic arrangement. The presence of a third OH group in the B ring does not enhance the effectiveness against aqueous phase radicals, except in the case of catechins; contributions to the antioxidant activity from hydroxyl

groups on the A ring are present in the absence of the dihydroxy structure in the B ring, predominantly [15].

1.3. Antioxidant systems

Endogenous antioxidants

Biological systems have evolved with endogenous defense mechanisms to help protect against free radical induced cell damage. Glutathione peroxidase, catalase, and superoxide dismutase are antioxidant enzymes, which metabolize toxic oxidative intermediates. They require micronutrient as cofactors such as selenium, iron, copper, zinc and manganese for optimum catalytic activity and effective antioxidant defense mechanisms [16,17,18]. Superoxide dismutase, catalase, and glutathione peroxidase are three primary enzymes, involved in direct elimination of active oxygen species (hydroxyl radical, superoxide radical, hydrogen peroxide) whereas glutathione reductase, glucose-6-phosphate dehydrogenase, and cytosolic GST are secondary enzymes, which help in the detoxification of ROS by decreasing peroxide levels or maintaining a steady supply of metabolic intermediates like glutathione and NADPH necessary for optimum functioning of the primary antioxidant enzymes [19,20] .

Glutathione, ascorbic acid, α-tocopherol, β-carotene, bilirubin, selenium, NADPH, butylhydroxyanisole (BHA), mannitol, benzoate, histidine peptide, the iron-bonding transferrin, dihydrolipoic acid, reduced CoQ_{10}, melatonin, uric acid, and plasma protein thiol, etc., as a whole play a homoeostatic or protective role against ROS produced during normal cellular metabolism and after active oxidation insult. Glutathione is the most significant component which directly

quenches ROS such as lipid peroxides and plays major role in xenobiotic metabolism. When an individual is exposed to high levels of xenobiotics, more glutathione is utilized for conjugation making it less available to serve as an antioxidant. It also maintains ascorbate (vitamin C) and alpha-tocopherol (vitamin E), in their reduced form, which also exert an antioxidant effect by quenching free radicals. Table I shows list of neutralizing antioxidants against ROS.

Table 1. Reactive oxygen species and their corresponding neutralizing antioxidants and also additional antioxidants

ROS	**Antioxidants**		**Antioxidants Exogenous**
	Direct role	**Indirect role**	
Hydroxyl radical	Glutathione peroxidase (cofactor selenium)	-	Vitamin C, lipoic acid
Lipid peroxide	Glutathione peroxidase (cofactor selenium)	-	Vitamin E, β-carotene
Superoxide radical	Superoxide dismutase (cofactor Cu/Zn/Mn)	Ceruloplasmin (Cu) Metallothionin (Cu) Albumin (Cu)	Vitamin C
Hydrogen peroxide	Catalase (cofactor iron)	Transferin (Fe) Ferritin (Fe) Myoglobin (Fe)	Vitamin C β-carotene Lipoic acid
Prooxidant/antioxidant equilibrium	Thiols (GSH,lipoic acid, N-acetylcysteine NADPH,NADH Ubiquinone)	Bilirubin Uric acid	Flavonoids

A number of other dietary antioxidants exist beyond the traditional vitamins collectively known as phytonutrients or phytochemicals which are being increasingly appreciated for their antioxidant activity, one example is flavonoids which are a group of polyphenolic compounds. These are widely found in plants as glucosylated derivatives. They are responsible for the different brilliant shades

such as blue, scarlet, and orange. They are found in leaves, flowers, fruits, seeds, nuts, grains, spices, different medicinal plants, and beverages such as wine, tea, and beer [21,22]. Table II shows food stuffs containing antioxidant constituents.

Table ii. Food stuffs containing antioxidant constituents

Food stuffs	**Constituents act as antioxidant**	**Food stuffs**	**Constituents act as antioxidant**
Citrus fruits/black tea	Quercetin, rutin, hesperetin, naringin	Cloves	Eugenol, cryophyllene
Tomato juice/green tea	Kaempferol	Fenugreek seeds	Diosgenin, sapogenin
Tomato Juice/vegetables	Fisetin,	Rosemary	Carnosol
Tomato Juice/vegetables	Myricetin	Mint (pudina)	Menthofuran, menthol
Propolis/fruits	Galangin	Garlic	Allyl sulfide
Soyabean/soy foods	Daidzein, daidzin,	Bel	Umbelliferone, marmalosin
Red clover	Biochanin A, formononetin,	Cereal products	Apigenin, luteolin
Fruits/vegetables	Cyanidin, cyanin,	Milk products	Casein, Vitamin-D
Fruits/vegetables	Chrysin	Fish	Cord oil (Vitamin-A)
Soyabeans	Genistein, genistin,	Eggs	Vitamin-A
Tomato	lycopene	Green and red chilli peppers	Capaisin

Cruciferous vegetables	Isothiocyanate, erucic acid	Black pipper	Piperine, piperidine, piperatine
Green and black tea	(+) - Catechin, (-) - Epicatechin (-) - Epigallocatechin	Cinamone	Eugenol, phellandrene
Ginko biloba	Bilobalide, ginkgolides	Saffron	Crocetin
Virgin olive oils	3, 4-dihydroxyphenylethanol (DOPET)	Ginger	Gingerol
Tulsi (basil)	Eugenol, nerol,	Karela	Vicine, momoridicine,
Ginseng	Ginsenosides	amla	Corilagin, ellagic acid, gallotanins
Walnuts, almonds	Oleic acid, alpha-linolenic acid, Saunf Anethole vitamins E, minerals	saunf	Anethole
Turmeric	Curcumin	shahtoot	Betulinic acid

2. Phenolic compounds

Phenolic compounds are a large, heterogeneous group of secondary plant metabolites that are widespread in the plant kingdom [23]. Phenolics display a vast variety of structures; here only flavonoids, tannins and phenolic acids are reviewed (**Figure 1**). The structural basis for all flavonoids is the flavone nucleus (2-phenyl-benzo-γ-pyrane) but, depending on the classification method, the

flavonoid group can be divided into several categories based on hydroxylation of the flavonoid nucleus as well as the linked sugar [24]. Essential flavonoid structures divided into eleven classes are presented in **Figure 2** [25]. "Tannins" is a general name for phenolic substances capable of tanning leather or precipitating gelatin from solution [26]. They can be divided into condensed proanthocyanidins, where the fundamental structural unit is the phenolic flavan-3-ol (catechin) nucleus, and into galloyl and hexahydroxydiphenoyl esters and their derivatives, gallotannins and ellagitannins [27], as shown in **Figure 1b**. The essential two groups of phenolic acids are hydroxybenzoic acids and hydroxycinnamic acids (**Figure 1c**), both of which are derived from non phenolic molecules benzoid and cinnamic acid, respectively [28].

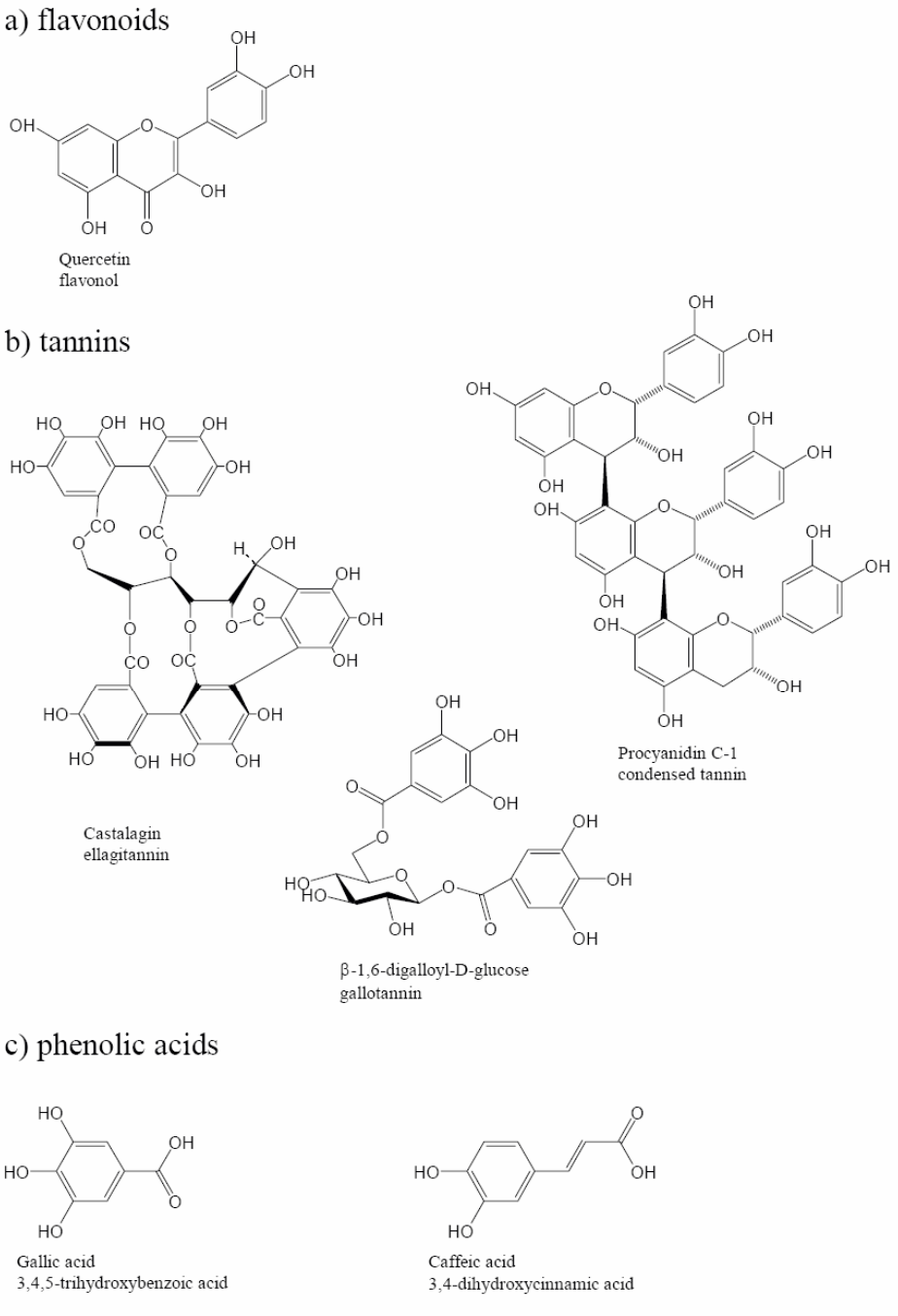


Figure 1 Typical structures of different groups of plant phenolics: **a**) flavonoids, **b**) tannins, **c**) phenolic acids.

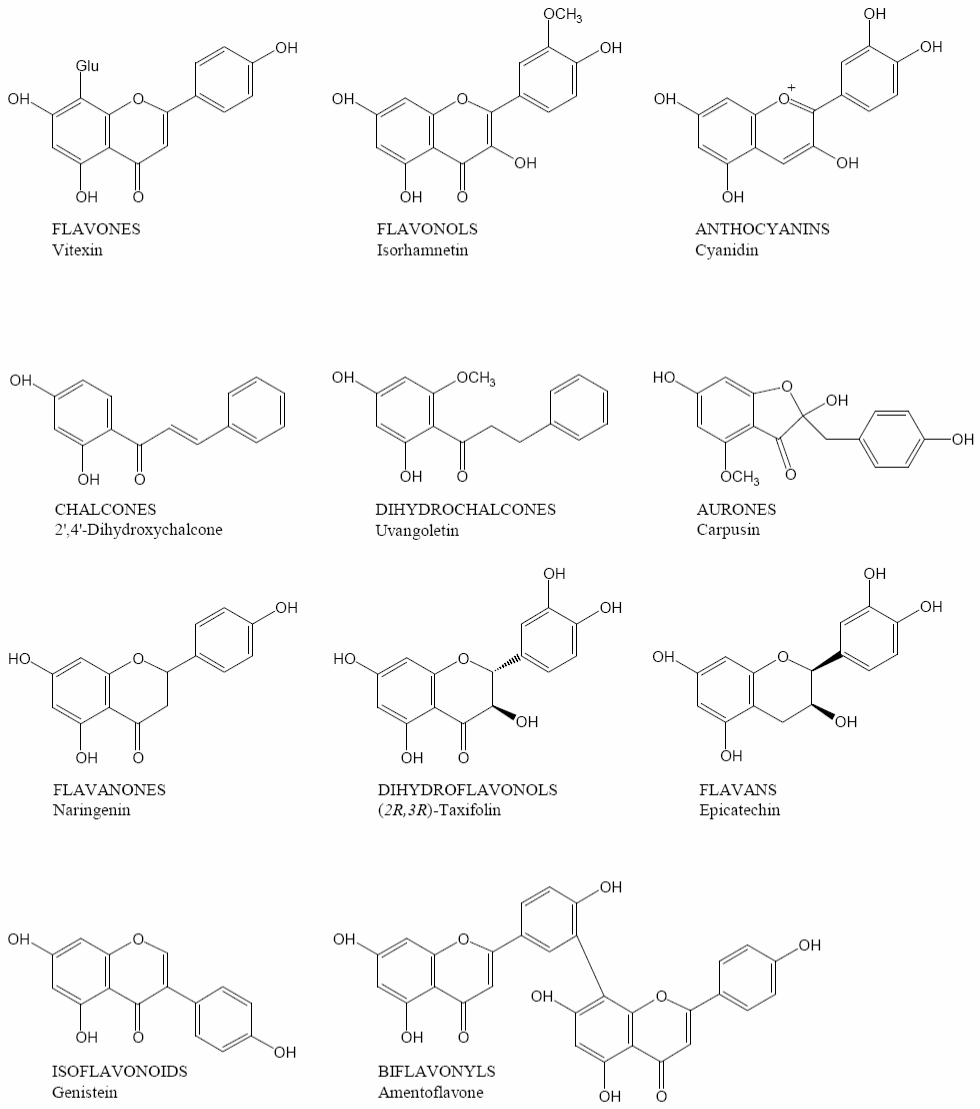


Figure 2 Typical structures of flavonoids belonging to the different subgroups.

3. Liver

The liver is a vital organ present in vertebrates and some other animals. It has a wide range of functions, including detoxification, protein synthesis, and production of biochemicals necessary for digestion. This organ plays a major role in metabolism and has a number of functions in the body, including glycogen storage, decomposition of red blood cells, plasma protein synthesis, hormone production and detoxification. It lies below the diaphragm in the thoracic region of the abdomen. It produces bile, an alkaline compound which aids in digestion, via the emulsification of lipids. It also performs and regulates a wide variety of high-volume biochemical reactions requiring highly specialized tissues, including the synthesis and breakdown of small and complex molecules, many of which are necessary for normal vital functions.

3.1. Hepatotoxicity caused by acetaminophen

It is known that acetaminophen (APAP), a potent analgesic and antipyretic drug with very few side effects at its usual therapeutic doses, can cause acute liver damage which leads to severe centrilobular hepatic necrosis, renal failure, and even death in humans and experimental animals when taken in overdoses [28,29, 30] or in moderate doses in combination with other drugs or alcohol [31].

An overdose of the analgesic drug acetaminophen (AAP) can lead to severe liver injury in humans and in experimental animals. Although intensely studied for more than 25 years, the mechanism of this injury is still not entirely clear. It is undisputed that the metabolism of a fraction of the AAP dose by the P450 system is the initial step of the injury process [32]. The product of this reaction is a

reactive metabolite, presumably *N*-acetyl-*p*-benzoquinone imine (NAPQI) [33,34], which is detoxified by glutathione [35]. However, if the formation of the reactive metabolite exceeds the capacity of liver glutathione, NAPQI will bind to cellular proteins. Over the years, a number of proteins were identified that were modified by NAPQI binding [36]. There is increasing evidence to suggest that protein binding is an initiating event of cell injury, which can be amplified through secondary processes [37]. One of these secondary effects of reactive metabolite formation and protein binding is mitochondrial dysfunction [38,39] which results in ATP depletion and oxidative stress [40,41]. Superoxide generated in mitochondria after AAP overdose can dismutate to form molecular oxygen and hydrogen peroxide, which is then reduced to water by glutathione peroxidase using electrons from GSH. The fact that mitochondrial glutathione disulfide (GSSG) levels increase substantially after AAP treatment is strong evidence for the increased hydrogen peroxide formation in mitochondria [42,43], although extra-mitochondrial sources of reactive oxygen cannot be excluded.

Studies with cultured mouse hepatocytes demonstrated that the oxidative stress, measured as increased 2,7-dichlorodihydrofluorescein diacetate (DCFH) fluorescence, precedes cell injury by several hours [44] consistent with an early increase in the GSSG-to-GSH ratio *in vivo* [45]. On the other hand, superoxide can react with nitric oxide (NO) to form the potent oxidant peroxynitrite [46]. The rate constant of the reaction between superoxide and NO is several times higher than the rate constant of superoxide dismutation with or without catalysis by superoxide dismutase [47]. Thus, an increased formation of superoxide in the

presence of equimolar Nitrotyrosine is a footprint for peroxynitrite formation [48]. Indeed, nitrotyrosine protein adducts can be detected in vascular endothelial cells and parenchymal cells after AAP overdose before cell injury, i.e., the loss of endothelial cell barrier function (hemorrhage) and ALT release, is observed [49, 50]. Administration of pharmacological doses of glutathione accelerated the recovery of mitochondrial glutathione levels, which effectively scavenged most of the peroxynitrite and protected against cell injury [51]. These data suggested that peroxynitrite is a critical mediator of AAP hepatotoxicity [51]. Both hydrogen peroxide (through hydroxyl radical formation by iron-catalyzed Fenton reaction) and peroxynitrite (through hydroxyl radical–like decomposition products) can initiate lipid peroxidation [52], which can lead to oncotic necrosis of liver cells. Furthermore, inhibition of peroxynitrite formation by inhibitors of nitric oxide synthase was associated with an increase in lipid peroxidation after AAP treatment [53]. Levels of NO can lead to increased formation of peroxynitrite in addition to hydrogen peroxide generation.

4. Aims of the study

The overall aim of the study was to investigate Brazilian plants rich in phenolics as a potential source of compounds possessing beneficial biological activities. The selection of plant material was based on the known use of the species as food or herbal remedies. The specific objectives of these studies are as under.

1. To provide a scientific base for the medicinal uses of these plants and validate their folkloric use in diseases arising from oxidative stress.
2. To evaluate the possible protection of crude extracts of studied plants against hepatotoxicity induced by paracetamol (acetaminophen) and study the active mechanism involved in the potential *In vivo* antioxidant and hepatoprotective activity of plants.
3. To characterize the phenolic composition of plant species with the most marked biological activity, by applying the most promising TLC/HPLC methods available.

5. General comment/General discussion

In recent years oxidative stress has been implicated in a variety of degenerative processes, diseases and syndromes. Some of these include atherosclerosis, myocardial infarction, stroke, ischemia/reperfusion injury, chronic and acute inflammatory conditions such as wound healing, central nervous system disorders such as forms of familial amyotrophic lateral sclerosis, Parkinson disease and Alzheimer's dementia and a number of age related disorders. The present study involves the antioxidant and hepatoprotective activity of four medicinal plants namely *Phyllanthus niruri*, *Solanum fastigiatum, Solidago microglossa and Baccharis trimera*. The antioxidant activity was evaluated using *in vitro* lipid peroxidation (TBARS), DPPH radical and iron chelation assays. Whereas, hepatoprotective activity of these plants were evaluated against paracetmaol induced liver damage in mice liver. Aqueous extract of these plants showed inhibition against lipid peroxidation induced by different pro-oxidants (10 μM FeSO4 and 5 μM sodium nitroprusside) in rat liver and brain homogenates. The extracts also lowered the formation of TBARS in egg yolk. *Phyllanthus niruri* significantly reduced the accumulation of lipid peroxides in a dose-dependent manner, from 2 to 10 μg/ml for iron and 10–80 μg/ml for SNP. However, the plant afforded greater protection against iron-induced lipid peroxidation than SNP, as it was active at low concentration. Increases in the formation of TBARS in iron(II) sulphate (10 μM)-induced oxidative stress, as compared to the normal, suggest possible damage of tissues with an overload of iron. Free iron in the cytosol and mitochondria can cause

considerable oxidative damage by increasing superoxide production, which can react with Fe(III) to regenerate Fe(II) that participates in the Fenton reaction [54]. Sodium nitroprusside is an anti-hypertensive drug which acts by relaxation of vascular smooth muscle; consequently it dilates peripheral arteries and veins. However, SNP has been reported to cause cytotoxicity through the release of cyanide and/or nitric oxide [55]. Protections offered by the extracts of these plants suggest that they may be useful in the treatment of liver and brain diseases. Metal chelation by phenolic compounds, in theory, could prevent iron-dependent lipid peroxidation in organisms by rendering iron inactive. Phenolic compounds of these plants showed a protective role against oxidative damage by sequestering iron (II) ions that may otherwise catalyze fenton-type reactions or participate in metal-catalyzed hydroperoxide decomposition reactions.

Varying concentrations of extracts were reacted with DPPH solution (0.1 mM in 95% EtOH) at room temperature. The absorbance at 517 nm was measured at various time points and used to calculate the percent scavenging of DPPH radicals. The IC_{50} of each sample was obtained by plotting the % scavenging of DPPH at the steady state (30 min) of the reaction against the corresponding fraction concentration. The IC_{50} is the concentration of the antioxidant needed to quench the DPPH by 50% under the experimental conditions. The IC_{50} value of *Solidago microglossa* (3.8± 0.1 µg/ml), *Phyllanthus niruri* (43.4 ± 1.45 µg/ml), *Solanum fastigiatum* (68.96±1.25 µg/ml) and *Baccharis trimera* (415.2±15.2 µg/ml) indicates that the order of antioxidant activity is *Solidago microglossa* > *Phyllantus niruri* > *Solanum fastigiatum* > *Baccharis trimera.*

Hepatoprotective activity of *Phyllanthus niruri, Solanum fastigiatum and Baccharis trimera* was shown by their ability to inhibit paracetamol-induced liver damage in mice. Liver injuries induced by acetaminophen are commonly used as models for the screening of hepatoprotective drugs [56]. Raised serum enzyme levels in intoxicated rats can be attributed to the damaged structural integrity of the liver [57], because these are cytoplasmic in location and are released into circulation after cellular damage [58]. The crude extract of *Phyllanthus niruri*, *Baccharis trimera* and *Solanum fastigiatum* used in this study seems to preserve the structural integrity of the hepatocellular membrane. This was evident from the protection provided to mice against the lethal dose of acetaminophen as well as a significant reduction in the acetaminophen induced rise in SGOT and SGPT levels in rats.

Paracetamol severely decreased the level of catalase in liver which was abolished by the treatment with the plants. The endogeneous enzymes such as the superoxide dismutase (SOD) catalyze the degradation of O_2^- to O_2 and H_2O_2, catalase (CAT) converts hydrogen peroxide into water and oxygen and glutathione peroxidase (GP_X) destroys toxic peroxide.

The comet assay was performed to evaluate the genotoxic potential of *Solidago microglossa.* Lymphocytes isolated from peripheral blood were treated with different concentrations (200, 500 and 1000 µg/ml) of extracts. The results revealed that the extract did not cause any significant (P<0.05) DNA damage at 200 and 500 µg/ml concentrations. However, there was a significant (P<0.05) but small increase in damage frequency at level 4 at very high concentration (1000

μg/ml). Regarding different *in vitro* methods the order of antioxidant activity was TBARS>DPPH>iron chelation.

Phytochemical analysis of the plants showed the presence of high contents of phenolics and flavonoids. Herbals and herbal extracts, which contain different classes of polyphenols, are very attractive, not only in modern phytotherapy, but also for the food industry, due to their use as preservatives. The total phenolic and flavonoid content was in the order of *Solanum fastigiatum*> *Solidago microglossa*> *Phyllanthus niruri*>*Baccharis timera*. The HPLC analysis showed the presence of quercetin (254.88±5.1 μg/ml), rutin (342.2±7.1 μg/ml) and gallic acid (2320.8±13.2 μg/ml) in the ethanolic extract of *Solidago microglossa.* Whereas, the HPLC analysis of aqueous extract of *Baccharis trimera* showed the presence of gallic acid (76.8±7.2 μg/ml) as a major compound whereas, rutin (20.1±2.5 μg/ml) and quercetin (10.1±1.4 μg/ml) relatively showed minor contribution.

6. Conclusions

In conclusion, the results of the present study demonstrated the high efficacy of the crude extract of *Phyllanthus niruri*, *Solanum fastigiatum*, *Solidago microglossa* and *Baccharis trimera* in free radical scavenging, inhibition of reactive oxygen species and lipid peroxidation which are related to the presence of phenolic compounds and effectiveness in the treatment of diseases among which the liver disease is the most important. The *Solanum fastigiatum* showed *in vivo* hepatoprotective activity against paracetamol induced liver damage and the plant material is relatively safe as is obvious by the lack of any symptom of acute toxicity at an oral dose of 4 g/kg and this study explains the traditional use of *Solanum fastigiatum* in hepatobiliary diseases. The ethanolic extract of *Solidago microglossa* inhibited 2-deoxy-D-ribose site-specific variant of the assay, we assume that they inhibit hydroxyl radical formation by chelating and deactivating iron ions. The high efficacy of these plants provides an economic alternative of great interest as the cost spent on the fractionation could be avoided if the crude extract itself has high antioxidant activity. These results may have implications in the use of the extract as a therapeutic agent in the prevention of oxidative stress related diseases. However, more detailed *in vivo* studies are needed to establish the safety and bioavailability of these plants.

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