

A close-up photograph of a hand holding a wooden mortar and pestle. The mortar is a reddish-brown ceramic bowl with a white mesh strainer inside, filled with small, dark, round seeds. The pestle is a thick, cylindrical wooden stick. The background is a dark, textured surface.

Journal of Medicinal Plant Research

Volume 8 Number 16, 25 April, 2014

ISSN 2009-9723



*Academic
Journals*

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Review

A review on pathophysiology of ischemic-reperfusion injury of heart and ameliorating role of flavonoids and polyphenols

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Received 13 January, 2014; Accepted 16 April, 2014

Ischemia-reperfusion (IR) syndrome is defined as injury caused by the restoration of coronary flow after a period of ischemia. The pathophysiology of ischemia-reperfusion injury involves cellular effect of ischemia, reactive oxygen species and inflammatory cascade. Flavonoids and polyphenols possess unique antioxidant properties and other protective activities which are beneficial for ischemia-reperfusion injury. It is found that flavonoids and polyphenols prevent production of reactive oxygen species and thereby inhibit oxidation of cellular components and also block propagation of oxidative reactions. They also increase the activity of endogenous antioxidant enzymes such as superoxide dismutase and catalase during ischemia-reperfusion injury. Flavonoids also possess anti-inflammatory, anti-platelet aggregation and vasodilatory effects through different mechanism. This review scrutinize to what extent flavonoids and polyphenols play a role in moderating ischemia-reperfusion mediated injury with special emphasis on pathophysiology of heart ischemic-reperfusion injury.

Key words: Ischemia, reperfusion, heart, flavonoids, polyphenols, antioxidant.

INTRODUCTION

Heart diseases are the major causes for most of the mortalities in the developed countries. World Health Organization (WHO, 1969) described heart disease as the greatest epidemic. In India, heart ailment has become the third greatest killer (Rajasekhar et al., 2004). According to the World Health Report (2002), cardio-vascular diseases (CVD) will be the largest cause of death and disability in India by 2020. Much of these are attributed to rapid acquisition adverse lifestyle which includes smoking, alcohol, physical in-activity, improper diet, stress, etc. Cardiovascular diseases killed nearly 17 million people

in 2011, which are 3 in every 10 deaths. Of these, 7 million people died of ischemic heart disease and 6.2 million from stroke (Figure 1 and Table 1).

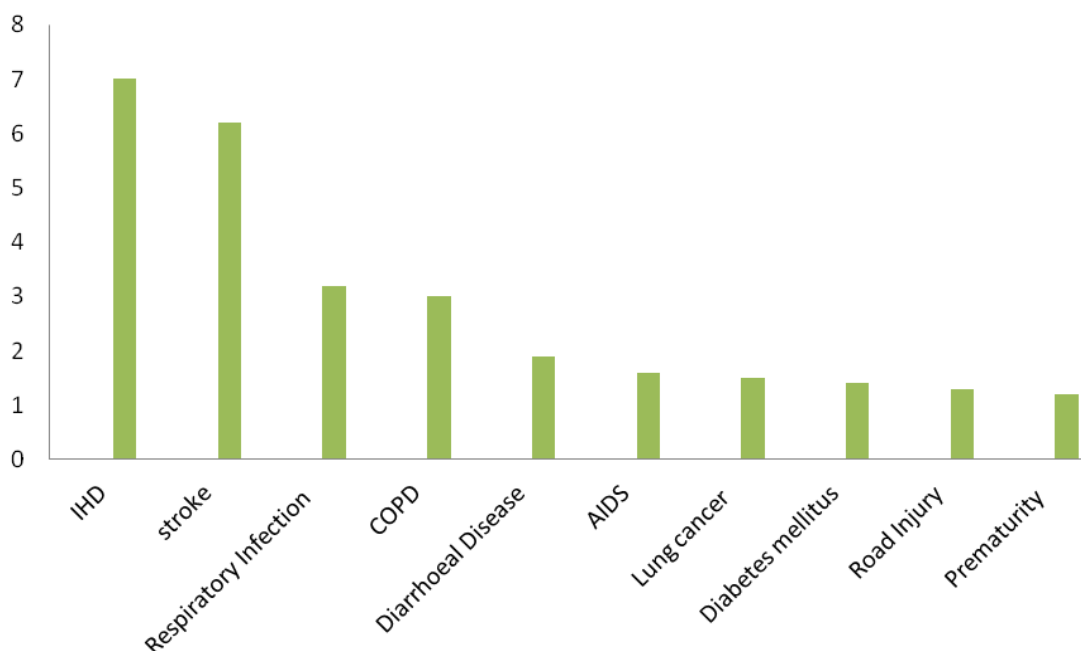
Ischemia comprises not only insufficiency of oxygen (hypoxia), but also reduced availability of nutrient and inadequate removal of metabolite. Ischemic heart disease (IHD) is due to narrowing or occlusion of one or more branches of coronary arteries. When a tissue is deprived of oxygenated blood flow followed by re-establishing the blood flow (reperfusion), the reintroduction of oxygen can result in tissue damage and is known as ischemic-

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Table 1. Top 10 leading causes of death in the world in the year 2011.

Disease	Death (million)
Ischemic heart disease (IHD)	7
Stroke	6.2
Respiratory infection	3.2
Chronic obstructive pulmonary disease (COPD)	3
Diarrhoeal disease	1.9
Acquired immune deficiency syndrome (AIDS)	1.6
Diabetes mellitus	1.5
Lung cancer	1.4
Road injury	1.3
Prematurity	1.2

**Figure 1.** Leading cause of death in the year 2011.

ischemic-reperfusion (IR) injury. In case of heart, IR injury is due to restoration of coronary blood flow after a period of myocardial ischemia. The main pathology for IR injury is over production of free radical, that is, reactive oxygen species (ROS) in the heart especially during the period of reperfusion. Reperfusion of ischemic heart is associated with vascular and micro-vascular injury, endothelial cell dysfunction, increased myocyte edema, necrosis, apoptosis and cardiac contractile dysfunction (Simon and Gregory, 1996). Conditions under which IR injury is encountered include the different forms of acute vascular occlusions (stroke, myocardial infarction) with their respective reperfusion strategies (thrombolytic therapy, angioplasty, operative revascularization, cardiopulmonary bypass, etc.) and major trauma/shock (Biagi et al., 2000).

The endogenous antioxidants such as glutathione peroxidases, superoxide dismutase and catalase act as primary defence mechanism whereas others, including vitamin E play a secondary role in attenuating the IR injury. But compared to other major organ, heart has low antioxidant defences and is highly vulnerable to free radical mediated damage (Pragada et al., 2004).

Several medicinal plants and their phytoconstituents (such as flavonoids and polyphenols) have been found to possess antioxidant properties and have beneficial effects in myocardial ischemia (MI) and reperfusion injury (Bhattacharya et al., 2002). A considerable number of these plant-based products have been widely used in India for the treatment of cardiovascular disease, as they are inexpensive, efficacious and safe (Mohanty et al., 2009).

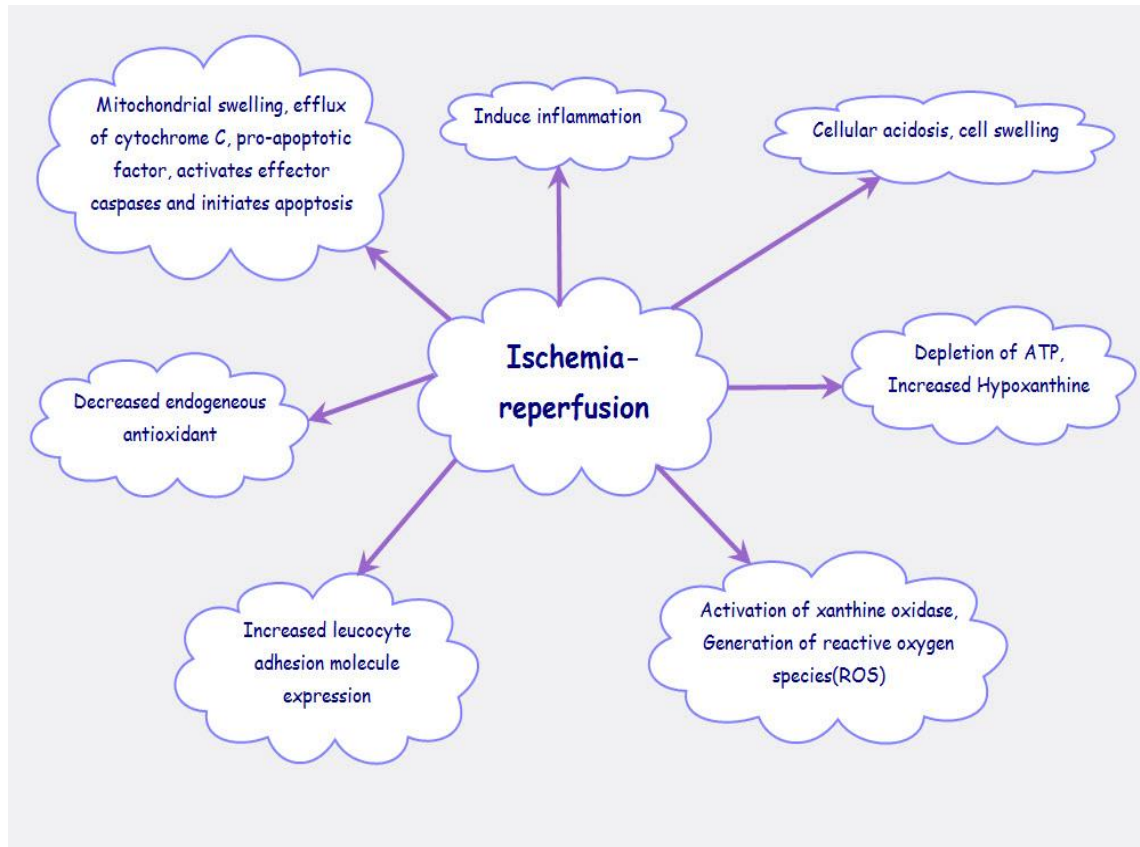


Figure 2. Pathophysiology of ischemia-reperfusion injury.

PATHOPHYSIOLOGY OF ISCHEMIC-REPERFUSION INJURY

Reperfusion of the previously ischemic myocardium is often followed by the detrimental changes in coronary arteries and myocardial tissues, which ultimately lead to cardiac dysfunction, known as ischemia-reperfusion injury (Figure 2).

Pathological changes associated with IR injury

Contractile dysfunction and reperfusion arrhythmias

The deleterious effects of ischemia-reperfusion injury are reversible contractile dysfunction known as myocardial stunning and impairment of blood flow at microvascular level. Myocardial stunning is the contractile dysfunction of heart that persists after reperfusion despite the absence of irreversible damage and despite restoration of normal or nearly normal coronary flow.

Reperfusion arrhythmias may be a cause of sudden death after relief of coronary ischemia and are frequent in patients undergoing thrombolytic therapy or myocardial surgical revascularization (Holger and Charles, 2004). Study demonstrates that reperfusion of the ischemic

myocardium in animals with normal coronaries often lead to the occurrence of ventricular tachycardia, ventricular fibrillation, or an accelerated idioventricular rhythm, particularly if performed abruptly after 15 to 20 min of ischemia. The occurrence of reperfusion arrhythmias may partly be a result of rapid and sudden alterations in ion concentrations within the ischemic region on reperfusion (Mahmood and Stephen, 2008).

No-reflow phenomenon

The detrimental effect of prolonged post-ischemic reperfusion is no-reflow phenomenon in which no blood flow occurs through coronary blood vessels due to increased leukocyte-endothelial cell adhesion, platelet-leukocyte aggregation, interstitial fluid accumulation and loss of endothelium-dependent vasorelaxation, which all together result in mechanical blood flow obstruction (Rezkalla and Kloner, 2002).

Energy depletion

Energy depletion is other harmful consequences of ischemia as a result of defective synthesis of adenosine

triphosphate (ATP) and degradation of energy rich phosphates, that is, ATP via adenosine diphosphate (ADP) and adenosine mono phosphate (AMP) to adenosine and finally hypoxanthine. Normally, hypoxanthine is converted to xanthine by the enzyme xanthine dehydrogenase in the presence of nicotinamide adenine dinucleotide (NAD). However, under ischemic conditions, xanthine dehydrogenase undergoes a conformational change to xanthine oxidase which is capable of producing highly ROS. This conformational change is also promoted by increased intracellular calcium (Ca^{2+}) (Rezkalla and Kloner, 2002; Buja, 2005).

Cellular acidosis, calcium overload and apoptosis

During ischemia, the reduced O_2 supply causes an increase in the rate of glycolysis, generating H^+ and lactate and decreasing intracellular pH (pHi). The Na^+/H^+ exchanger (NHE) overloads the cytosol with Na^+ as the excess H^+ are extruded, causing the reversal of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, which extrudes excess Na^+ , but overloads the cytosol with Ca^{2+} . The depletion of ATP during ischemia prevents the activity of pumps such as the Na^+/K^+ ATPase, as well as active Ca^{2+} excretion which prevents the re-establishment of normal cellular ionic homeostasis (Buja, 2005; Karmazyn et al., 1999). Furthermore, there is an increase in ROS production if the first minute of reperfusion is very high as O_2 is re-introduced into damaged mitochondria. Mitochondrial Ca^{2+} overload and increased ROS can result in opening of the mitochondrial permeability transition pore and initiates the translocation of BAX (apoptosis regulator also known as Bcl-2 like protein), from the cytosol to the outer mitochondrial membrane. This causes mitochondrial swelling and induces the efflux of cytochrome C and other pro-apoptotic factor via opening of the permeability transition pore into the cytosol where cytochrome C activates effector caspases and initiates apoptosis (Halestrap et al., 2004).

ROS generation and lipid peroxidation

Under physiological conditions, 95% of oxygen is reduced in the mitochondrion to H_2O via tetravalent reduction without any free radical intermediates, whereas 5% is reduced by univalent pathway in which free radicals like superoxide anion ($\text{O}_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are produced and are safely metabolized to H_2O by dismutase, catalase and the glutathione peroxidase system. With ischemia, antioxidant defenses become eroded and thus increasingly generates the highly destructive hydroxyl radical (OH^{\cdot}) and superoxide ($\text{O}_2^{\cdot-}$) ion that causes direct damage to cellular membranes as well as proteins and induces lipid peroxidation (Braunersreuther and Jaquet, 2012). Following restoration

restoration of oxygen supply, the production of ROS by dysfunctional mitochondria rises dramatically and directly damage cellular membranes through lipid peroxidation (Simpson and Lucchesi, 1987).

Leukocyte activation

ROS stimulate leukocyte activation and chemotaxis by activating synthesis of eicosanoids such as thromboxane A_2 and leukotriene B_4 (Buja, 2005). ROS also stimulates leukocyte adhesion molecule and cytokine gene expression via activation of transcription factors such as nuclear factor κB (NF κB) (Zingarelli et al., 2003). Leukocyte activation release proteases and elastases, which result in increased microvascular permeability, edema, thrombosis, and cell death. Various signaling systems such as tumor necrosis factor- α (TNF- α), mitogen activated protein kinase (MAPK), caspases, interleukin-1 (IL-1) and IL-6 are also involved in the pathophysiology of IR injury (Simpson and Lucchesi, 1987; Toyokuni, 1999).

Nitric oxide

Nitric oxide (NO) can also be a mediator of tissue damage during ischemia-reperfusion injury, as it reacts with the abundantly prevalent superoxide anion to form peroxynitrite (ONOO^-) and subsequently dissociates into the highly cytotoxic species NO_2 and OH^{\cdot} . However, as NO also exerts cytoprotective effects, the exact role of NOS enzymes in IR injury is yet to be confirmed (Schulz et al., 2004).

Acute inflammatory response and chemotaxis

Cardiac ischemia-reperfusion injury triggers an acute inflammatory response in which neutrophils via chemotactic attraction infiltrate the myocardium and worsen the condition of the already injured tissue. Endothelial cells, in response to specific stimuli like ROS release chemoattractants. These includes leukotriene B_4 , monocyte chemoattractants protein (MCP), adhesion molecules such as intercellular adhesion molecule 1 (ICAM 1), vascular cell adhesion molecules (VCAM) and selectins, leading to neutrophil attraction (Kukielka et al., 1993), sequestration and adhesion to the microvasculature. Accumulation and sequestration of neutrophils in the coronary microcirculation can lead to the occlusion of the microvasculature and thereby interferes with blood flow in the reperfused region (Seal and Gewertz, 2005).

Activation of compliment system

Ischemic-reperfusion results in local activation of

compliment system and leads to production of compliment factors C3a, C5a, and membrane attack complex (MAC). C5a exerts numerous pro-inflammatory effects such as chemotaxis of neutrophils, release of proteases, production of oxygen radicals which may further amplify the inflammatory response by initiating production of TNF, IL-1, and IL-6 and monocyte chemo-attractants protein (MCP-1). Predominant role of C5b-9 is also indicated in IR mediated tissue injury (Kukielka et al., 1993; Seal and Gewertz, 2005).

AMELIORATING ROLE OF FLAVONOIDS AND POLYPHENOLS

Polyphenols are natural substances with variable phenolic structures and are elevated in vegetables, fruits, grains, bark, roots, tea, and wine. Polyphenols are the most abundant antioxidant in the diet (Manach et al., 2004). Their total dietary intake could be as high as 100 mg/day to 1 g/day, which is much higher than all other classes of phytochemicals and dietary anti-oxidants even much higher than Vitamin C and vitamin E (Rice-Evans, 2001). About 8000 different polyphenols are known to be widely present in plants, and their structure can range from simple compounds to highly polymerized structures, such as tannins (Ghasemi et al., 2009). It is reported that antioxidant activity fruits and vegetables significantly increases with the presence of high concentration of total polyphenols content (Fantinelli et al., 2005).

Flavonoids are a subgroup of the more extended family of polyphenols with a basic structure containing two benzene rings with a pyrane ring in the middle. Flavonoids are outstanding antioxidants and because of their antioxidant activity as well as their abundance in fruit and vegetables, they may partly contribute to the currently-known health benefits of plant foods (Aneja et al., 2004; Ikizler et al., 2007; Modun et al., 2003).

Experimental findings of some flavonoids and polyphenols against ischemic-reperfusion injury

(1) The administration of crataegus flavonoid by oral route protects the brain against delayed cell death caused by ischemia-reperfusion injury and also found to increase the antioxidant level in the brain (Zhang et al., 2004).

(2) The garlic-derived flavonoids also have a protective effect against ischemic brain injury. The neuroprotective effect of garlic might be associated with control of the free-radical burst and preservation of antioxidant enzyme activity (Aguilera et al., 2010).

(3) Epigallocatechin-3-gallate (EGCG), that is, the most prominent catechin polyphenols found in green tea is beneficial for the treatment of reperfusion induced myocardial damage. Experimental evidence showed that

EGCG reduced myocardial damage by decreasing plasma IL-6, creatine phosphokinase levels and myeloperoxidase activity in rats. The beneficial effect of EGCG was also said to be associated with reduction of nuclear factor- κ B and activator protein-1 DNA binding (Aneja et al., 2004).

(4) Quercetin when administered before ischemia reduced malondialdehyde levels in heart tissues after reperfusion (Ikizler et al., 2007). Similarly, 30 days feeding of rats with red grapes also attenuated formation of malondialdehyde in ischemic-reperfused hearts (Pataki et al., 2002).

(5) Salvianolic acid A, constituent of *Salvia miltiorrhiza* has potent antioxidant activity against peroxidized damage to biomembranes. This beneficial effect protects vascular walls from oxidation, inflammation, thrombus formation, etc., (Zhang et al., 2010).

PLAUSIBLE PROTECTIVE MECHANISMS OF FLAVONOIDS AND POLYPHENOLS

Antioxidant and free radical scavenging activity

Flavonoids, polyphenols and their metabolites display antioxidant activity (Pietta, 2000), also potent scavengers (Chun et al., 2003) of ROS such as superoxide, peroxide radicals, and peroxy nitrite. Their ability to increase the plasma antioxidant status and preservation of erythrocyte membrane polyunsaturated fatty acids has been proved experimentally. They inhibit the lipid peroxidation, thereby prevent peroxidized damage of biomembranes and formation of malondialdehyde. Flavonoids exert cardiovascular protection by decreasing oxidative stress and increasing NO bioavailability (Maulik et al., 1996). Polyphenols, especially, the flavonols such as kaempferol, quercetin and their derivatives may inhibit the oxidation of low density lipoprotein (LDL) cholesterol, reduce platelet aggregation, or reduce ischemic damage. Polyphenols such as apple polyphenols acts by suppressing mitochondrial superoxide production, thus prevent mitochondrial damage. Flavonoids at relatively low concentrations can be important antioxidants in microenvironments that are less accessible to vitamin C and vitamin E. Interestingly, it has been suggested that specific flavonoids upon binding metals may behave as a superoxide dismutase, scavenging superoxide more potently than the parent flavonoids, while devoid of catalytic activity for the Fenton conversion of hydrogen peroxide to hydroxyl radicals (Vanisha et al., 2010; Malesev and Kuntic, 2007) (Figure 3).

Antioxidant in microenvironment and intermediate antioxidant

Flavonoids are proposed to act as intermediate antioxidants

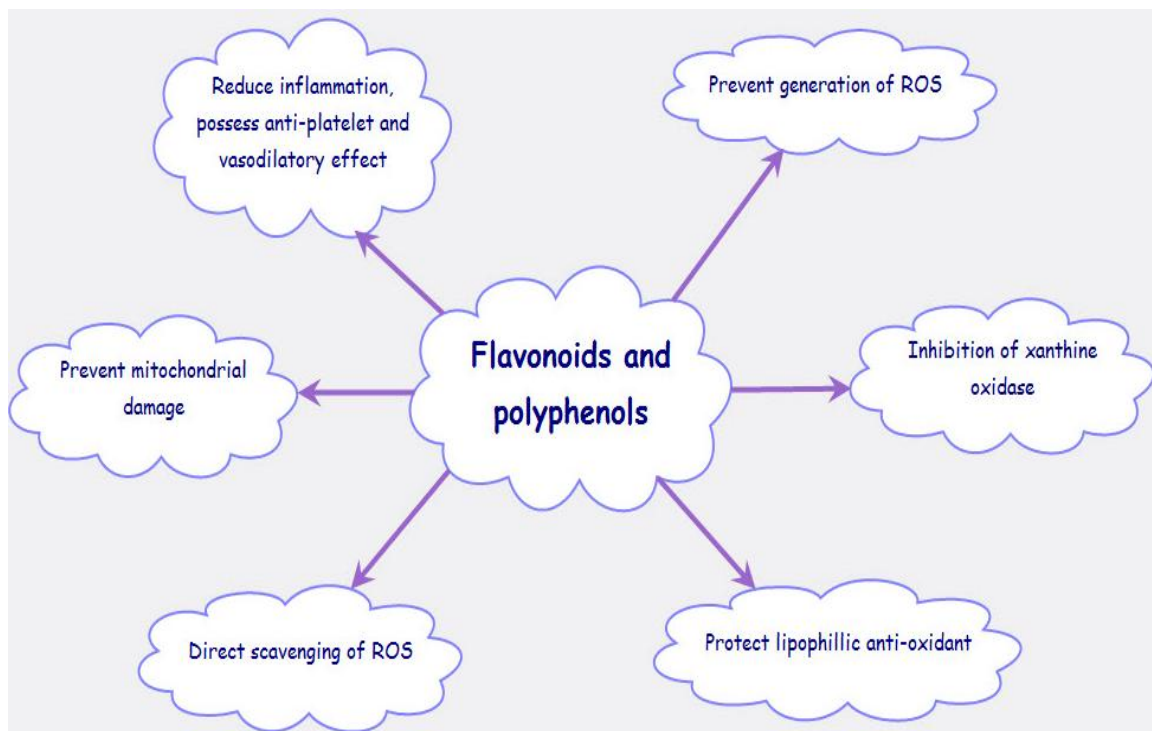


Figure 3. Plausible protective mechanisms of flavonoids and polyphenols.

antioxidants, where protecting lipophilic antioxidants (Vitamin E) and being protected by hydrophilic antioxidants (Vitamin C). Flavonoids also possess the ability to chelate iron ions known to catalyze many free radical-generating processes. This property probably also contributes to their antioxidant effectiveness (Lotito and Fraga, 2000) (Figure 3).

Inhibition of xanthine oxidase and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase

These may be other mechanisms by which flavonoids at physiological concentrations can mitigate ischemia-reperfusion injury (Cos et al., 1998). Several flavonoids including luteolin, apigenin, quercetin, myricetin, and kaempferol have been shown to inhibit xanthine oxidase. Inhibition of the NADPH oxidase of endothelial cells has recently been proposed as a mechanism by which catechins improve vascular function, which could be of benefit in protecting against ischemia-reperfusion injury (Schewe et al., 2008) (Figure 3).

Vasodilatory effect

A variety of flavonoids and polyphenols have shown the capacity to dilate blood vessels (vasodilatation). Their

mechanism of action is various and may be exerted in endothelium-dependent and/or independent manners. The endothelium-dependent relaxation effect of polyphenols is mediated by nitric oxide. Flavonoids may also promote vasorelaxation by stimulating production of prostacyclins by endothelial cells (Ajay et al., 2003; Novakovic et al., 2006) (Figure 3).

Anti-inflammatory and anti-platelet effect

Flavonoids inhibit the enzymes involved in eicosanoids pathways, including phospholipase A₂ (Kim et al., 2004), cyclooxygenases and lipoxygenases, thus limits the production of inflammatory mediators such as prostaglandins and leukotrienes (Kim et al., 1998). Flavonoids and polyphenols can inhibit the expression of inflammatory mediators such as ICAM-1, by acting on NFκB activation. Flavonoids can also inhibit production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and interferon-γ (Rimbach et al., 2001). Flavonoids have also been shown to inhibit platelet activation and aggregation (Gallego et al., 2007). The anti-platelet effect of flavonoids may be because of increased production of prostacyclin or by increased cyclic adenosine monophosphate (cAMP) through inhibition of phosphodiesterases responsible for degradation of cAMP (Bertelli et al., 1995) (Figure 3).

Inhibition of matrix metalloproteinases

Matrix metalloproteinases (MMP) are a family of proteases that play a major role in protein degradation and tissue remodeling. It is confirmed experimentally that flavonoids, at physiologically relevant concentrations, inhibit matrix metalloproteinases (especially two members of this enzyme family, namely MMP-2 and -9) (Ende and Gebhardt, 2004) (Figure 3).

CONCLUSION

Research on the effect of dietary polyphenols and flavonoids on human health developed considerably in past 10 years. It strongly supports a role for polyphenols and flavonoids in the prevention of cardiovascular disease. It has become clear and evident that the flavonoids and polyphenols exert myocardial protective effects via antioxidant activities, preservation of nitric oxide, anti-inflammatory activities and modulation of matrix metalloproteinases.

There have been many studies in various systems on the polyphenols and flavonoids, however more depth study about their source and the way they protect the heart from ischemia-reperfusion injury is highly appreciated and warranted. This will establish much more effective correlations between isolated polyphenols, flavonoids and their cardioprotective effect.

CONFLICT OF INTEREST

The authors have declared that there no conflict of competing interest.

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Full Length Research Paper

***In vitro* anti-arthritic and thrombolytic activities of methanolic extract of *Protium serratum* leaves**

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Received 14 March, 2014; Accepted 13 April, 2014

The present study was engineered to find out the anti-arthritic and thrombolytic activity of methanolic extract of *Protium serratum* by using *in vitro* method. Methanolic extract of *P. serratum* was assessed with the inhibition of protein denaturation model which was used to evaluate anti-arthritic potential and this extract assessed with human blood to evaluate thrombolytic effect. The extract showed remarkable anti-arthritic activity which was evaluated by the percentages of inhibition of protein denaturation in different concentration of plant extract compared to diclofenac sodium, a reference drug. The maximum percentage inhibition of protein denaturation was observed as 83.94% at 1000 µg/ml. It has significant thrombolytic effect compared to streptokinase. During assay for thrombolytic activity, the methanolic extract of *P. serratum* revealed $59.653 \pm 8.626\%$ lysis of clot, while standard streptokinase (SK) and water used as positive and negative controls, demonstrated 72.835 ± 5.702 and $2.725 \pm 0.983\%$ lysis of clot, respectively. These findings demonstrate that the leaves extract of *P. serratum* have excellent anti-arthritic and thrombolytic effect.

Key words: *Protium serratum*, anti-arthritic, thrombolytic, % lysis of clot, protein denaturation.

INTRODUCTION

Arthritis is an auto immune disorder characterized by pain, swelling and stiffness. Its prevalence depends upon age. It occurs more frequently in women than in men. It is an inflammation of synovial joint due to immune mediated response. All anti inflammatory drugs are not anti arthritic because it does not suppress T-cell and B-cell mediated response. Rheumatoid arthritis (RA) is an autoimmune disorder characterized by synovial proliferation, inflammation, subsequent destruction like deformity of joints or

destruction of cartilage and bone (Firestein, 2003). RA is the most common inflammatory joint disease in humans and has long been classified among the autoimmune diseases in which skeletal complications start with focal erosion of cartilage followed by marginal and subchondral bone loss. Extended joint destruction with ankylosis and generalized bone loss are characteristic for late complications (Feldmann et al., 1996). These long-term skeletal complications have serious consequences as they can lead

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not only to painful joint deformities but also to progressive functional disability and increased mortality rates (Pincus et al., 1993).

The production of auto antigens in certain arthritic diseases may be due to *in vivo* denaturation of proteins (Brown et al., 1968). The mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding (Grant et al., 1970). So, by controlling the production of auto antigen and inhibiting denaturation of protein and membrane lysis in rheumatic disease leads to anti-arthritic activity. Hence, inhibition of protein denaturation and membrane lysis were taken as a measure of the *in vitro* anti-arthritic activity (Volluri et al., 2011).

Formation of blood clots is one of the vital reasons of blood circulation problem. Thrombi or emboli can lodge in a blood vessel and block the flow of blood in that location depriving tissues of normal blood flow and oxygen. This can result in damage, destruction (infarction), or even death of the tissues (necrosis) in that area (Thrombus, 2011). A blood clot (thrombus) is formed from fibrinogen by thrombin and is lysed by plasmin which is activated from plasminogen by tissue plasminogen activator (tPA). Fibrinolytic drugs has been used to dissolve thrombi in acutely occluded coronary arteries there by restoring blood supply to ischemic myocardium to limit necrosis and to improve prognosis (Laurence, 1992).

Streptokinase is an antigenic thrombolytic agent used for the treatment of acute myocardial infarction. It reduces mortality as effectively as the nonantigenic alteplase in most infarct patients while having the advantages of being much less expensive. Tissue-type plasminogen activator (tPA) is generally preferred as being effective and safer than either urokinase or streptokinase type activators. All available thrombolytic agents still have significant shortcomings, including the need for large doses to be maximally effective, limited fibrin specificity and a significant associated bleeding tendency. Because of the shortcomings of the available thrombolytic drugs, attempts are underway to develop improved recombinant variants of these drugs (Nicolini et al., 1992; Adams et al., 1991; Lijnen et al., 1991; Marder, 1993; Wu et al., 2001).

The plant kingdom represents an enormous reservoir of biologically active compounds with various chemical structures and protective/disease preventive properties (phytochemicals). Nearly 50% of drugs used in medicine are of plant origin and only a small fraction of plants with medicinal activity has been assayed. Therefore much current research have been devoted to the phytochemical investigation of higher plants which have ethno-botanical information associated with them. The phytochemicals isolated are then screened for different types of biological activity like thrombolytic potentials (Harborne, 1998). Herbal preparations are used potential source of medicine since ancient times to maintain health and regain healthy state of mind. Herbs showing thrombolytic activity have been studied and some significant observations

have been reported (Basta et al., 2004).

Protium serratum (Syn. *Bursera serrata*) belonging to the family Burseraceae is an evergreen resinous small sized tree, distributed mostly in hilly areas, deciduous and semi-evergreen forests in Bangladesh, Native India and the Philippines. It yields aromatic oil and edible fruits (Huq et al., 1987). *Protium* species showed anti-inflammatory (Carretero et al., 2008), anti-tumor (McDoniel et al., 1972) and agglutinating and immobilizing activities (Huacuja et al., 1990). Terpenoids and coumarin were analyzed by Ara et al. (2009). Since there is no scientific report for anti-arthritic and thrombolytic potential of *P. serratum* leaf extract, the present study was an attempt to evaluate the anti-arthritic and thrombolytic effect by *in vitro* analysis.

MATERIALS AND METHODS

Plant

The leaves of *P. serratum* were collected from Bandarban district of Chittagong hill tracts region of Bangladesh in 2013 and authenticated by Dr. Shaikh Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh.

Extracts preparation

The collected plant was washed thoroughly with water and air dried for a week at 35 to 40°C and pulverized in electric grinder. The obtained powder was successively extracted in methanol (55 to 60°C). The extracts were made to dry by using rotary evaporator under reduced pressure.

In vitro test analysis

In vitro anti-arthritic activity

For the evaluation *in vitro* anti-arthritic activity of *P. serratum*, the method used was "inhibition of protein denaturation" (Mishra et al., 2011; Lavanya et al., 2010; Singh et al., 2011; Chippada et al., 2011) using diclofenac sodium a standard. The test solution (0.5 ml) consists of 0.45 ml of bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of test solution (methanolic extract of *P. serratum*). The test control solution (0.5 ml) consists of 0.45 ml of bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of distilled water. Product control (0.5 ml) consists of 0.45 ml of distilled water and 0.05 ml of test solution. Standard solution (0.5 ml) consists of 0.45 ml of bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of diclofenac sodium. Various concentrations (62.5, 125, 250, 500, 1000 µg/ml) of methanolic extract of *P. serratum* (MEPS) and diclofenac sodium (standard) were taken, respectively. All the solutions were adjusted to pH 6.3 using 1 N HCl. The samples were incubated at 37°C for 20 min and the temperature was increased to keep the samples at 57°C for 3 min. After cooling, 2.5 ml of phosphate buffer was add to the previous solutions. The absorbance was measured using UV-Visible spectrophotometer at 416 nm. The control represents 100% protein denaturation. The results were compared with diclofenac sodium. The percentage inhibition of protein denaturation of different concentrations is tabulated in Table 1. The percentage inhibition of protein denaturation can be calculated as:

Table 1. Percentage inhibition of protein denaturation.

Concentration ($\mu\text{g/ml}$)	Percentage inhibition in protein denaturation	
	MEPS (Test solution)	Diclofenac sodium
62.5	45.21	68.05
125	54.29	72.65
250	62.24	79.21
500	71.09	85.04
1000	83.05	92.94

Table 2. Effect of herbal extracts on *in vitro* clot lysis.

Extracts/Drugs	Mean \pm SD (% of clot lysis)
Water (Negative Control)	2.725 \pm 0.983%
Streptokinase (Positive Control)	72.835 \pm 5.702%*
Methanolic extract of <i>P. serratum</i> (L.)	59.653 \pm 8.626%*

Statistical representation of the effective clot lysis percentage by herbal preparations, positive thrombolytic control (Streptokinase) and negative control (sterile distilled water) done by paired t-test analysis; % clot lysis is represented as mean \pm S.D. and * $p < 0.0001$, significant compared to control.

$$\% \text{ of Inhibition} = \frac{[100 - (\text{OD of test solution} - \text{OD of product control})]}{\times 100}$$

Where OD = optical density

The control represents 100% protein denaturation. The results were compared with diclofenac sodium.

***In-vitro* thrombolytic test**

The thrombolytic activity of this extractive was evaluated by the *in vitro* thrombolytic test (Prasad et al., 2006) using streptokinase as standard. The dry crude extract (10 mg) was suspended in 10 ml of distilled water and it was kept overnight. Then the soluble supernatant was decanted and filtered. Aliquots (5 ml) of venous blood were drawn from healthy volunteers which were distributed in five different pre weighed sterile micro centrifuge tube (1 ml/tube) and incubated at 37°C for 45 min. After clot formation, the serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (Clot weight = weight of clot containing tube – weight of tube alone). To each micro-centrifuge tube containing pre-weighed clot, 100 μl aqueous solutions of different partitionates along with the crude extract was added separately. As a positive control, 100 μl of streptokinase (SK) and as a negative non thrombolytic control, 100 μl of distilled water were separately added to the control tubes. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. After incubation, the released of fluid was removed and tubes were again weighed to observe the difference in weight after clot disruption. The differences in weights taken before and after clot lysis were expressed as percentage of clot lysis as shown. % of clot lysis = (weight of released clot / clot weight) \times 100

Statistical analysis

The significance between % clot lysis by herbal extract by means of weight difference was tested by the paired t-test analysis. Data are expressed as mean \pm standard deviation.

RESULTS

Different concentrations of methanolic extract of *P. serratum* and diclofenac sodium were tested for anti-arthritic activity and found significant percentage inhibition in protein denaturation (Table 1). The methanolic extract of *P. serratum* (MEPS) also showed significant thrombolytic activity with 59.653 \pm 8.626% (Table 2) lysis of clot. The positive control (streptokinase) showed 72.835 \pm 5.702% and negative non thrombolytic control (distilled water) showed 2.725 \pm 0.983% lysis of clot.

DISCUSSION

In *in vitro* protein denaturation test, the MEPS have shown significant activity at various concentrations and its effect was compared with the standard drug diclofenac sodium. The maximum percentage inhibition of protein denaturation was observed as 83.94% at 1000 $\mu\text{g/ml}$ which was close to the percentage of inhibition of diclofenac sodium (92.94%) as shown in Table 1. In *in vitro* thrombolytic test, *P. serratum* possesses good thrombolytic activity in comparison with streptokinase. So that, we may assume that these extracts can be considered as a potential source of natural anti-arthritic as well as thrombolytic agent. In context of the discussion, it would be interesting to investigate the causative components/mechanism for clot lysis by these plant extracts and for protein denaturation activity. This is only a preliminary study and to make final comment, the extract should thoroughly be investigated phytochemically and pharmacologically to exploit their medicinal and pharmaceutical potentialities.

ACKNOWLEDGEMENT

We would like to acknowledge that this research was supported by the Department of Pharmacy, International Islamic University Chittagong (IIUC), Chittagong-4203, Bangladesh. We would like to thank Mr. Md. Rafikul Islam, Assistant professor, IIUC for providing procedures and equipments.

Conflict of Interest

The authors have declared that there no conflict of competing interest.

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Full Length Research Paper

Management of indigenous medicinal plants in Nigeria using phenological information

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Received 18 June, 2013; Accepted 28 March, 2014

Available information on medicinal plants indicates habitat loss and has heightened the need for more proactive conservation strategies. Conservation efforts in this direction resulted in an eco-pharmacological survey in the West African sub-region by three countries (Nigeria, Ghana and Republic of Benin) to assess frequently used medicinal plants. The result of the socio-economic study based on utilization pattern regionally was used to produce a list of ten topmost frequently used and mentioned medicinal plants regionally and was subsequently adopted for the eco-pharmacological study in Nigeria. The list was super-imposed on a vegetation map of Nigeria for study sites selection in the species range. Selection criteria were based on the presence of at least one or more members of the medicinal plants in each location. Subsequently these plants were monitored range-wide for phenological behaviors for two seasons (dry and raining seasons) for two consecutive years. Findings indicated that the ten medicinal plants belonged to nine taxonomic families and are represented in the three plant habits. Flowering was majorly a dry season event (November to February) extending to early rains (March to April) in the species of the southern range (lowland and derived ecozones). Fruiting was typical of early rains increasing northwards (March to July). For short duration flowering species (*Pcynanthus angolensis*, *Alstonea bonnie* and *Rauvolfia vomitoria*), fruiting occurred late in dry season (December to February). Mean flowering duration ranged between (9.45 ± 1.73 to 45.68 ± 4.77) days, while mean fruiting duration ranged between (15.22 ± 2.15 to 145.87 ± 8.59) days. Phenological charts were used to depict the phenological trends for the different species. The provision of this information offers a useful tool-kit for medicinal plants genetic resources monitoring, management and appropriate conservation strategies in Nigeria.

Key words: Indigenous medicinal plants, eco-pharmacological survey, phenology, conservation.

INTRODUCTION

African wealth of biological resources and plant genetic resources in particular is a critical element in alleviating

poverty, ensuring food security and developing new medicines (Van Wyke, 2008). In addition, they possess

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immeasurable socio-cultural values and significance (Gillespie et al., 2004). Mgeni (1991) opined that with the unique diversity of plant and animal life, tropical rain forest represents biological renewable resources of food, medicine and fuel if well managed. Within the natural forests in Nigeria abound several valuable non-timber resources of edible and highly nutritive plants whose fruits, leaves, stems, twigs, barks and roots are of high medicinal values (Ugbogu and Odewo, 2004; Oni, 2010). Medicinal plants no doubt, will continue to play significant roles in both rural and peri-urban health care services as evident in the number of herbal practitioners in Nigeria today (pers. com). It had also enjoyed steady and popularity without any religious or ethnic barriers as evident in the number of modern alternative practitioners in many big cities of Nigeria (World Health Organization (WHO), 2001).

They are also important for pharmacological research and drug development either as direct therapeutic agents or as sources of templates for the synthesis of drugs (Farnsworth et al., 1985). According to World Health Organization (2004), despite all advances made in orthodox medicine, traditional medicine will continue to gain renewed interest in health care services of Nigerians. This may be attributable to increased awareness in the potential and curative ability of these alternative medicines and in particular the various shortcomings revealed for several synthetic drugs (Ugbogu and Odewo, 2004). According to Gbile and Adesina (1986), herbs usually serve as the repository materials and have been acknowledged to be generally safe with minimum side effects. The potential of the Nigerian flora as a veritable source for pharmaceutical and other therapeutic materials have been emphasized (Gbile and Adesina, 1986; Anselm-Adodo, 2004). The dwindling economic fortunes, political instability and high cost of orthodox medicines have also forced many people to exploit various plant species for their health service (Odebiyi and Ogunjobi, 2003).

However despite these potentials, many of these valuable plant species are fast disappearing and the current rate of genetic erosion through loss of species, varieties and habitat considered as alarming, has heightened the need to adopt more proactive steps in the conservation and use of these imperative resources (Bada and Popoola, 2005; Food and Agricultural Organization (FAO), 2005). Most of these losses had been traced to rapid rate of natural forest conversion to mono species plantations, commercial agriculture and other economic activities (Olajide, 2003; Owonubi and Otegbeye, 2004; Bada and Popoola, 2005). A major challenge in natural forest resources management in Nigeria is the continuous decline in stock over the years (Ajakaiye, 2001). Muul (1993) indicated that many of the old folks who possess knowledge and information on the

use and conservation of most of these medicinal plants are gradually dying without adequate documentation of their knowledge.

Unlike several tropical timber species and multipurpose tree species (Oni and Fagbenro, 2000) which had received significant domestication research attention, there is dearth of information on several aspects of medicinal plants including their reproductive biology. According to Van Wkye (2008), typically, studies on medicinal plants have focused on the bioactivity of its chemical macronutrients, ethnobotany, pharmacology and taxonomy while information on reproductive biology activities remains scanty in literatures. Perhaps this accounts for why many of them still remain in the wild state. The general opinion especially among collectors is that there will also be enough in the wild; unfortunately the trend is changing. Recent ecological survey indicated various threats (natural and anthropogenic forces) leading to low species biodiversity and poor natural regeneration. In recent times there had been an upward surge in medicinal plants exploitation without corresponding domestication efforts.

Developing appropriate conservation and silvicultural programme for these categories of plants required an initial adequate base line data on their flowering and fruiting behaviors across their range to assist adequate germplasm collections and *ex situ* conservation programme. In Nigeria, onset of flowering in *Parkia biglobosa* and *Vitellaria paradoxa* increased in a south-north direction (Hall et al., 1996). The present study therefore attempts to provide additional information in this regard for future germplasm collection programme, silvicultural studies and *ex situ* conservation strategies for the selected medical plants.

MATERIALS AND METHOD

The study involved three West African countries namely; Nigeria, Ghana and Republic of Benin. At the onset of the study, an initial socio-economic study was carried out by the three collaborating countries to identify frequently used medicinal plants species in the region. After the survey, the data were analyzed using simple descriptive statistics of frequency distribution and the ten most frequently mentioned across the three countries were harmonized and adopted for use in the phenological study and was subsequently used to develop a common protocol of study (Table 1). In Nigeria, the adopted and working list of medicinal plants were reconciled with locations where previous socio-economic survey data were collected and on that basis, the various locations were plotted on a vegetation map of Nigeria to produce a distribution map for the ten selected medicinal plants.

The different medicinal plants coincided with different agro-ecological/vegetation zones of Nigeria (Lowland Rainforest zone, Derived savanna, Guinea savanna and Sudan savanna) as shown in Figure 1. As a way of providing insight into distinct climatic differences among the various agro-ecological zones, a summary of their main physical features and climatic variables were as summarized in Table 2.

Table 1. The ten selected medicinal plants investigated for phenological behaviors in Nigeria.

S/No	Medicinal plants investigated in the study
1	<i>Alstonea bonnie</i>
2	<i>Khaya senegalensis</i>
3	<i>Kigelia Africana</i>
4	<i>Morinda lucida</i>
5	<i>Pycnathus angolensis</i>
6	<i>Rauvolfia vomitoria</i>
7	<i>Securidaca longpenduculata</i>
8	<i>Tamarindus indica</i>
9	<i>Vitellaria paradoxa</i>
10	<i>Zanthoxylum xanthoxyloides</i>

Source: Socio-economic survey (2009).

Table 2. The bio-physical characteristics of the selected study sites in relation to their agro-ecological zones in Nigeria.

Agro-ecological zone	Study site	Latitude (°N)	Longitude (°E)	Altitude (m)	Annual rainfall (mm)	Rainfall pattern
Lowland rain forest	Benin	6 19'	5 41'	120	1440	Bi-modal
	Ekpoma	7 23'	3 56'	120	1450	
	Ibadan	6 20'	5 40'	264	1340	
	Ido/Eruwa axis	7 23	3 54'	264	1340	
Derived savannah	Eruwa	7 35'	3 25'	264	1340	Bi-modal
	Olokemeji	7 35'	3 25'	200	1222	
	Igana-Okeho axis	7 50'	3 55'	102	1040	
	Saki axis	8 41'	3 23	106	1040	
Guinea savannah	Ibilo	7 50'	6 07'	52	1180	Mono-modal
	Okene-Kabba axis	7 49'	6 44'	41	1184	
	Lokoja axis	7 44	8 35'	114	1280	
	Makurdi axis	7 26'	6 04'	120	1180	
Sudan savannah	Kano	12 05'	8 35'	172	886	Mono-modal
	Taraba	8 38'	11 08'	168	920	

Description of the different ecological zones for the study in Nigeria

The Lowland rainforest is located south of the derived savannah. It constitutes a belt varying in width between 50 to 250 km, located inland from the coast and lies below latitude 8°N to the southwest (Figure 1). It corresponds to areas where rainfall generally exceeds 1,300 mm. Humidity is generally high and there is a long wet season lasting from 8 to 10 months. The zone supports high forest vegetation and only about 2% of the zone is estimated to be undisturbed forest. The Derived savanna which is the next ecological zone is a transitional forest-savannah mosaic occurring north of the lowland rain-forest belt, extending from 8° 30'N in the

West to 6° 40'N in the East. It covers an area of approximately 75,707 km² (about 8% of the country), of which constituted forest reserves, covers only 3208 km² (3.34%) of the total area of forest reserves in the country (Figure 1). The Guinea savannah zone covers a land area of about 459,033 km² and is located within latitudes 9 and 12°N of Nigeria. This zone represents Nigeria's broadest vegetation zone constituting approximately one half of the country's total land area. The Sudan savanna zone includes areas that lie above latitude 10°N of the country and is characterized by arid and semi arid conditions. The natural vegetation of the zone has been modified over most of the area by several centuries of human activities, including intensive cultivation, livestock grazing, annual fires and desert encroachment.

Table 3. The ten selected medicinal plants investigated for phenological behavior in Nigeria.

S/No	Selected medicinal plants Taxonomy	Family	Habit
1	<i>Alstonea bonnie</i> De Willd	Apocynaceae	Tree
2	<i>Khaya senegalensis</i> (Desr) A. Juss	Meliaceae	Tree
3	<i>Kigelia africana</i> (Lam.) Benth.	Bignoniaceae	Shrub
4	<i>Morinda lucida</i> Benth.	Rubiaceae	Shrub
5	<i>Pycnathus angolensis</i> (Welw.) Warb.	Myristicaceae	Tree
6	<i>Rauvolfia vomitoria</i> Afzel.	Apocynaceae	Shrub
7	<i>Securidaca longpenduculata</i> Fresen.	Polygalaceae	Herb
8	<i>Tamarindus indica</i> L.	Fabaceae	Tree
9	<i>Vitellaria paradoxa</i> C.F Gaertn.	Sapotaceae	Tree
10	<i>Zanthoxylum xanthoxyloides</i> (Lam.) Zep & Timler	Rutaceae	Tree

Protocol of work for the eco-phenological survey and data collection procedure

In addition to the initial distribution map developed for the medicinal plants, a review of literature was carried out to further obtain additional information on the natural distribution of the ten selected medicinal plants. Results obtained were used to upgrade the medicinal plants distribution map across the different ecological zones. The distribution map was therefore used as guide for the field work. At the onset of the field work, each of the four ecological zone selected was divided into two main sampling sites in east-west directions and study sites selection were based on adequate representation of the different medicinal plant species in each location on ecozone basis. The final sampling sites selection was based on the presence of at least one or more of these medicinal plants in each site. Thereafter, field observations periods were stratified into dry and raining seasons (November to March and April to October, respectively) as typical of climatic conditions in Nigeria. At each sampling site, individual medicinal plant species encountered was assessed for their reproductive biology status (phenology) during each season for the following reproductive characters: (i) onset of flowering, (ii) duration of floral buds opening, (iii) development of flowers to fruits and (iv) fruit maturation duration. These observations were carried out over a two year period for the different medicinal plants. Data collected were subjected to analysis of variance and the means separated by least significant difference (LSD) for each phenological parameter. The observed trends for the flowering and fruiting periods for each medicinal plant species investigated were subsequently depicted using phenological charts for seasonal variations description. The different medicinal plants were also classified into their taxonomic families as well as plant habits. Findings for the various activities were as reported in the result section.

RESULTS AND DISCUSSION

Distribution of the ten selected medicinal plants by taxonomic family and habit

The present study indicated that the ten different medicinal plants belonged to nine taxonomic families and only two members (*Alstonea bonnie* and *Rauvolfia vomitoria*) belonged to the same taxonomic family

(Apocynaceae) (Table 3). The different medicinal plants were also represented in the three major plant habits (Trees, shrub and herbs) (Table 3). The plant habits distribution indicated that six of the medicinal plants are trees, three were shrubs and only one (*Securidaca longpenduculata*) belonged to herb habitat (Table 3). The fewer number of plants belonging to the same taxonomic family may be explained based on their utility selection criterion rather than their natural co-existence which made the present findings different from the work of Ugboogu and Akinyemi (2004) who studied the ethnobotany and conservation of Ribako strict natural reserve in Northern Nigeria.

Flowering behaviors among the different medicinal plants in Nigeria

Generally, flowering behaviors varied greatly among the ten different medicinal plants investigated perhaps due to their taxa differences. Majority of them were observed flowering during the early to late dry season (November to February) in Nigeria and continues till early rains (March to April) especially for the lowland and derived ecozones species (*P. angolenses*, *Morinda lucida*, *Rauvolfia vomitoria* and *A. bonnie*). For typical savannah species (*Vitellaria paradoxa*, *Khaya senegalensis* and *Tamarindus indica*), flowering were observed during the months of May to July coinciding with late dry season to early rains in those agro-ecological zones and this process increased northwards in the species range. Two of the medicinal plant species (*M. lucida* and *R. vomitoria*) demonstrated broad ecological amplitude being conspicuously found in Lowland rainforest and Derived savanna zones. They were observed flowering in the late dry season (January to February) in the lowland rain forest zone (Ibadan) and in the late raining season (September to October) in its Derived savanna range (Eruwa and Saki axis).

Table 4. Mean duration of flowering to fruit for the selected ten different medicinal plants in Nigeria.

S/No	Species	Mean no of days to flowering (days)±sd	Mean Fruit set period (days)±sd
1	<i>Alstonea bonnie</i>	9.45±0.35	15.22±1.15
2	<i>Kigelia qfricana</i>	12.7±0.31	43.56±2.0.26
3	<i>Khaya senegalensis</i>	8.42±0.33	34.39±1.18
4	<i>Morinda lucida</i>	17.25±1.72	27.58±2.26
5	<i>Pycnathus angolenses</i>	27.34±2.35	21.44±1.28
6	<i>Rauvolfia vomitoria</i>	15.68±1.55	25.36±3.22
7	<i>Tamarindus indica</i>	14.25±0.78	31.45±2.37
8	<i>S. longipedunculata</i>	8.35±0.38	15.95±1.51
9	<i>Vitellaria paradoxa</i>	45.68±3.77	145.87±7.86
10	<i>Z. xanthoxyloides</i>	16.68±2.15	18.52±0.43

Onset of flowering for most of the medicinal plants tends to vary with latitude in a south-north direction. Onset of flowering for the same species varies by about 2 to 4 weeks interval in a south-north direction across the species range. Hopkins (1983) observed that within a given species population, two periods of flowering may occur, each lasting 3 to 4 weeks with good synchrony between trees. Pettet (1977) observed that onset of flowering within a distance of 80 km apart (Zaria to Kaduna) in Nigeria varied by seven days and it was suggested that it may be photo-periodically controlled and presumably modified by other environmental factors (Hopkins, 1983; Fatubarin, 1987). In Ghana, Hall et al. (1996) observed similar trend in *V. paradoxa*. *K. senegalensis* was observed flowering between November to February across its range in derived savanna zone in Nigeria (Makurdi and Okene). El-Amin (1990) observed similar trends in the species in Sudan.

In similar studies in Burkina Faso, *K. senegalensis* was observed flowering in November to December while in Guinea (November to February), Sudan (February to March) and (August to December) in Cote' Ivoire (Coalition for National Science Funding (CNSF), 2003). Flowering behaviours in relation to the geographical locations of these countries also agreed with the present findings. For many of the medicinal plants, new flush (emergence of young leaves) tends to be associated with onset of flowering in many locations where data were collected. This is in agreement with the work of Abbiw (1990) and (CNSF, 2003) on *A. bonnie*, *M. lucida* and *R. vomitoria* which are typical lowland rainforest species were observed to have extended to derived savanna agro-ecological zones as a result of climate change and anthropogenic activities and onset of flowerings were found to be closely associated with new flushes. *T. indica*, a savanna species, found in the northern limit of rainforest zone was observed flowering in January to March (Eruwa-Olokemeji axis). However, for the typical

savannah medicinal plants (*V. paradoxa*, *K. senegalensis*, *T. indica* and *Z. xanthoxyloides*), flowering was majorly a late dry season event (November to January) in their derived savanna range but extending northwards of the Guinea and Sudan savanna range. This observation was in agreement with the work of Hall et al. (1996) on *V. paradoxa* and *K. senegalensis* (CNSF, 2003). However, *S. longipedunculata* typical of the derived savannah ecozone (Eruwa and Okeho-Saki) axis had two flowering periods (February to April) and (June to July) though the second flowering period and was heavier perhaps due increase soil moisture, as this period coincided with the rainfall peak. Instances of two flowering periods for indigenous tree species had been indicated in Nigeria by Ladipo et al. (1990).

Duration of flowering period also varied greatly among the different species ranging between 8.35 ± 1.18 days in *S. longipedunculata* to 45.68 ± 4.77 days in *V. paradoxa*. Flowering in *P. angolenses* was very short and rather inconsistent and could not be determined accurately apart from the fact that most of the trees were extremely too tall to allow for effective flowering behaviors monitoring. In the species typical locations, corresponding to the lowland rainforest zone (Auchi and Ibadan), flowering was already over by February when the trees were examined, however two individual trees observed within the University of Ibadan campus showed evidence of flowering in late October to November. This finding tends to agree with the report of Abbiw (1990) in Ghana. Table 4 summarized the mean flowering and fruiting durations for the ten different medicinal plants investigated in Nigeria.

Fruiting behaviours for the selected ten medicinal plants in Nigeria

Fruiting behaviors among the different medicinal plants

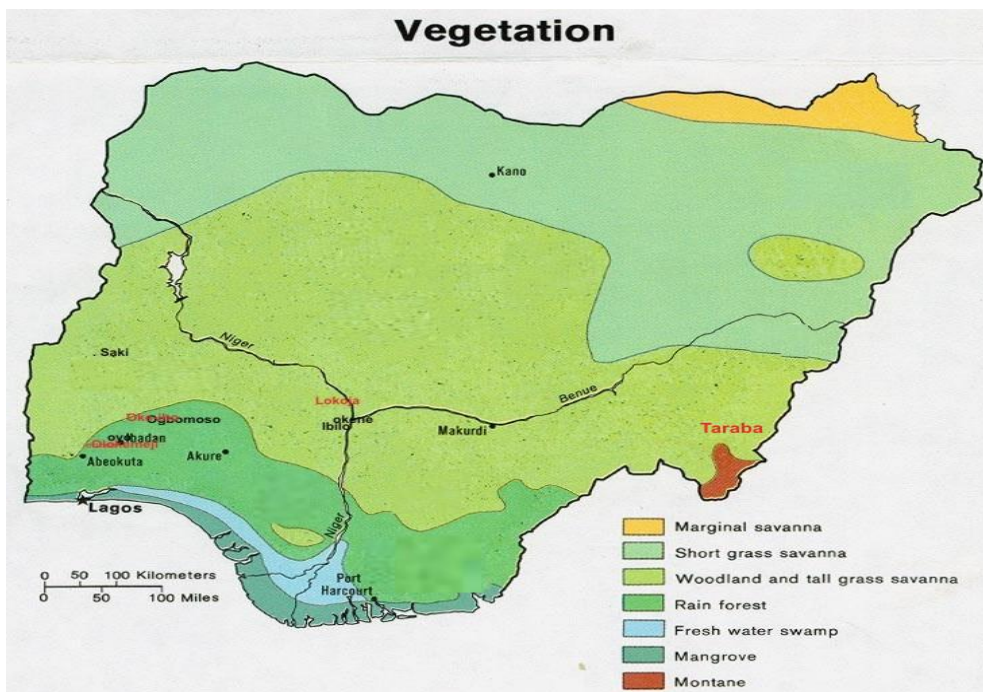


Figure 1. Vegetation map of Nigeria showing the sampling sites.

Source of flowering information	Flowering records in relation to months in Nigeria												Co-ordinates of the location			
	J	F	M	A	M	J	J	A	S	O	N	D				
Eruwa axis																7° 24' N 3° 29'E
Benin axis																6° 19' N 5° 41'E
Saki axis																8°39'N 3° 25'E

Figure 2. Flowering information of *S. longipedunculata* in Nigeria in relation to periods of the year across the various ecological zones.

tend to follow similar pattern like the flowering; though some degrees of overlaps were observed in some of the species. In several locations within the range of *M. lucida* and *R. vomitoria*, flowering and fruiting were observed simultaneously in the early rains (March to April). For instance, along Igana-Okeho axis (Derived savanna), fruiting was observed in March in *R. vomitoria*. Similar reports had been indicated for these species in some tropical West African countries (Burkill, 1985). Onset of flowering to fruit set duration also differs greatly for the individual medicinal plants investigated (Table 3). It ranged between (15.22 ± 1.15) days in *A. bonnie* to (145.87 ± 6.89) days in *V. paradoxa* (Table 3). The varying duration observed tends to be influenced both by varying climatic factors as well as the differences in the tree species.

Similar observations had been indicated by Fatuabrin (1987) in Nigeria. *K. africana* was observed in fruits between (February to March) in Ibillo (Figure 4) while, *S. longipedunculata* was observed fruiting in Eruwa (June to July) both belonging to the derived savanna. This is in agreement with previous observations made for these species in selected countries in West African (FAO, 1985; El- Amin, 1990). The fruiting trends and varying patterns across the different locations also increased in a south-north direction like the flowering behaviors which further corroborated the work of Hall et al. (1996) on *V. paradoxa*. The varying durations of flowering and fruiting for the different medicinal plants were as depicted in phenological charts across the different species range to enable comprehensive seasonality variations to be clearly observed (Figures 1 to 20).

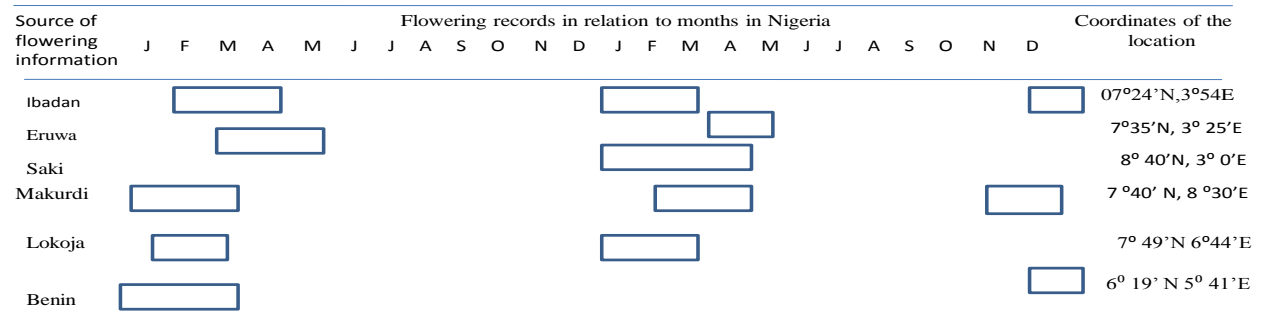


Figure 3. Flowering information in *K. senegalensis* in relation to periods of the year across the various ecological zones in Nigeria.

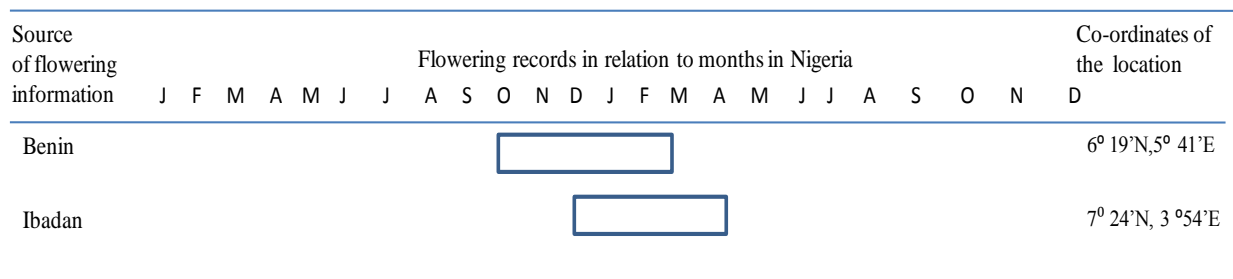


Figure 4. Flowering information in *Alstonia bonnie* in Nigeria in relation to periods of the year across the various ecological zones.

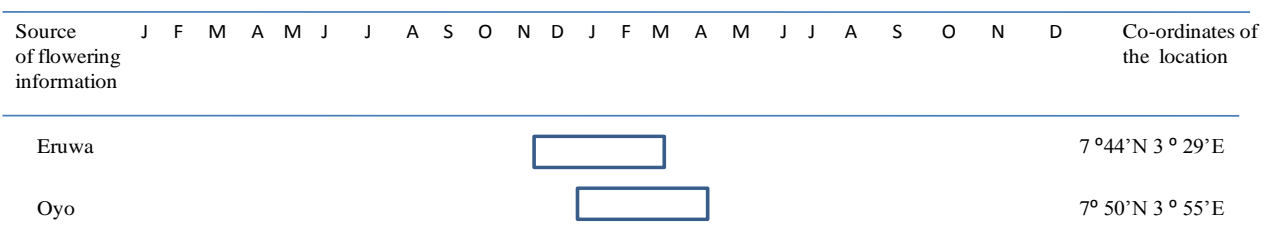


Figure 5. Flowering information in *Tamarindus indica* in Nigeria in relation to periods of the year across the various ecological zones.

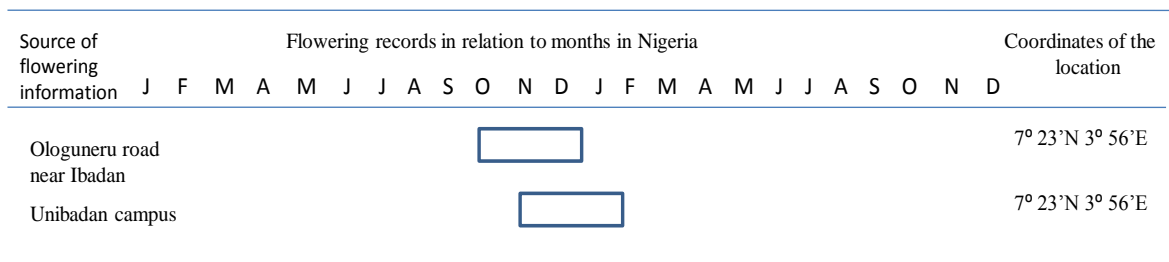


Figure 6. Flowering information in *Pychnatus angolensi* in Nigeria in relation to periods of the year across the various ecological zones.

Source of flowering information	Flowering records in relation to months in Nigeria												Coordinates of the location											
	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D
Eruwa																								7° 3' 24" N 3° 29' E
Benin axis																								6° 19' N 5° 41' E
Kabba																								73° 50' N 6° 07' E

Figure 7. Flowering information in *Kigelia africana* in Nigeria in relation to periods of the year across the various ecological zones.

Source of flowering information	Flowering records in relation to months in Nigeria												Coordinates of the location											
	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D
Olokemeji																								7° 24' N 3° 29' E
Eruwa																								7° 24' N 3° 29' E
Makurdi																								7° 44' N 8° 35' E

Figure 8. Flowering information in *Zanthoxylum xanthoxyloides* in Nigeria in relation to periods of the year across the various ecological zones.

Source of flowering information	Flowering records in relation to months in Nigeria												Coordinates of the location											
	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D
Eruwa																								7° 24' N 3° 29' E
Saki																								8° 39' N 3° 25' E
Makurdi																								7° 44' N 8° 35' E
Kabba																								7° 50' N 6° 07' E
Kaso																								12° 00' N 8° 31' E

Figure 9. Flowering information in *Zanthoxylum xanthoxyloides* in Nigeria in relation to periods of the year across the various ecological zones.

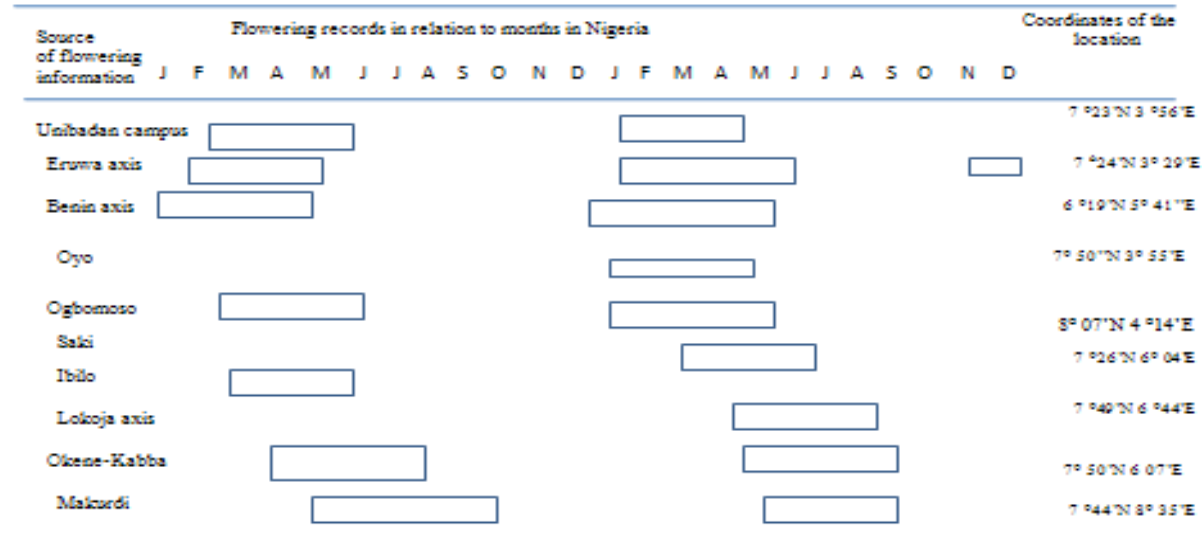


Figure 10. Flowering information in *Rauvolfia vomitoria* in Nigeria in relation to periods of the year across the various ecological zones.

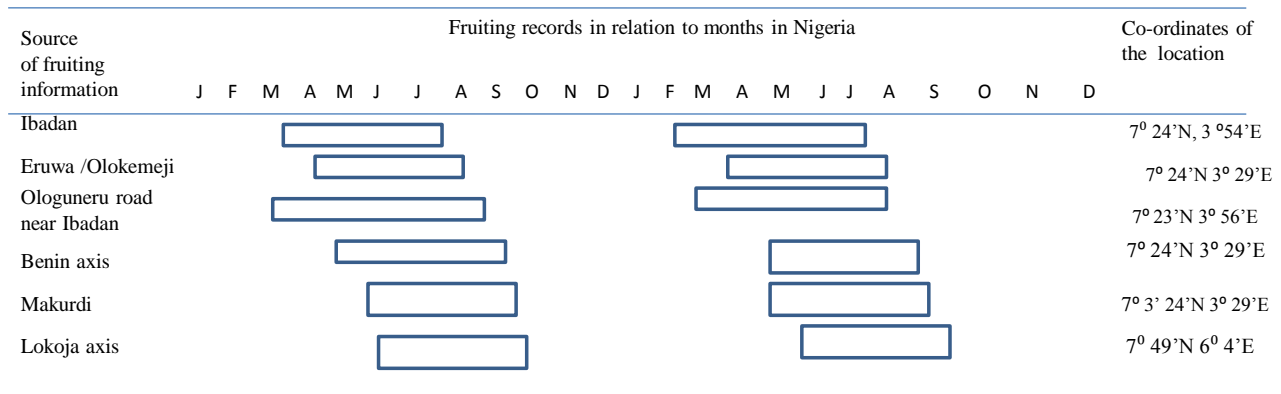


Figure 11. Fruiting information in *M. lucida* in Nigeria in relation to periods of the year across the various ecological zones.

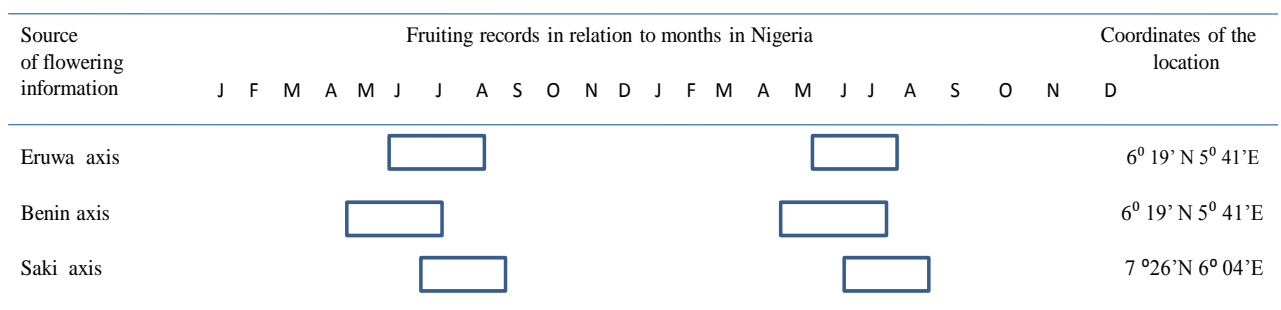


Figure 12. Fruiting information in *S. longipedunculata* in Nigeria in relation to periods of the year across the various ecological zones.

Source of fruiting information	Fruiting records in relation to months in Nigeria												Co-ordinates of the location					
	J	F	M	A	M	J	J	A	S	O	N	D						
Unibadan				[]														7° 24'N, 3 °54'E
Ologuneru –Eruwa road				[]														7° 24'N, 3 °54'E
Benin axis					[]													6° 19'N,5° 41'E

Figure 13. Fruiting information in *Alstonia bonnei* in Nigeria in relation to periods of the year across the various ecological zones.

Source of fruiting information	Fruiting records in relation to months in Nigeria												Co-ordinates of the location					
	J	F	M	A	M	J	J	A	S	O	N	D						
Eruwa				[]														6° 19' N 5° 41' E
Benin axis				[]														6° 19'N,5° 41' E
Kabba				[]														72°50'N 6° 07'E

Figure 14. Fruiting information in *K.africana* in Nigeria in relation to periods of the year across the various ecological zones.

Source of fruiting information	Fruiting records in relation to months in Nigeria												Co-ordinates of the location					
	J	F	M	A	M	J	J	A	S	O	N	D						
Eruwa				[]														7 °44'N 3 ° 29'E
Oyo				[]														7° 50'N 3 ° 55'E

Figure 15. Fruiting information in *Tamarindus indica* in Nigeria in relation to periods of the year across the various ecological zones.

Source of fruiting information	Fruiting records in relation to months in Nigeria												Co-ordinates of the location					
	J	F	M	A	M	J	J	A	S	O	N	D						
Ibadan				[]														07°24'N,3°54E
Eruwa					[]													7 °44'N 3 ° 29'E
Saki						[]												8° 39'N 3° 25'E
Lokoja						[]												7° 49'N 6° 44'E
Makurdi						[]												7 °44'N 8° 35'E
Benin				[]														6° 19' N 5° 41'E

Figure 16. Fruiting information in *Khaya senegalensis* in Nigeria in relation to periods of the year across the various ecological zones.

Source of fruiting information	Fruiting records in relation to months in Nigeria																								Co-ordinates of the location
	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	
Ologuneru road near Ibadan	[]																			[]					7° 23'N 3° 56'E
Unibadan campus	[]																			[]					7° 23'N 3° 56'E

Figure 17. Fruiting information in *Pychnatus angolensis* in Nigeria in relation to periods of the year across the various ecological zones.

Source of fruiting information	Fruiting records in relation to months in Nigeria																								Co-ordinates of the location
	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	
Olokemeji	[]																			[]					7° 24'N 3° 29'E
Eruwa	[]																			[]					7° 24'N 3° 29'E
Makurdi	[]																			[]					7° 44'N 8° 35'E

Figure 18. Fruiting information in *Zhanthoxylum xanthoxyloides* in Nigeria in relation to periods of the year across the various ecological zones.

Source of fruiting information	Fruiting records in relation to months in Nigeria																								Coordinates of the location
	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	
Eruwa	[]																			[]					7° 24'N 3° 29'E
Saki	[]																			[]					8° 39'N 3° 25'E
Makurdi	[]																			[]					7° 44'N 8° 35'E
Kabba	[]																			[]					7° 50'N 6° 07'E
Kano	[]																			[]					12° 00'N 8° 31'E

Figure 19. Fruiting information in *Vitellaria paradoxa* in Nigeria in relation to periods of the year across the various ecological zones.

Conclusion

Phenological study of this nature cutting across several medicinal plants of ethnobotanical and ethno-pharmacological importance are particularly very vital in the development of any sustainable management programme for genetic resources conservation and use of these different medicinal plants. This is particularly useful as majority of these medicinal plants still come from the wild, while domestication efforts and *ex-situ* conservation efforts still remain low range-wide. A realistic starting point in addressing this shortcoming is the availability of holistic information on the flowering and

fruiting behaviours among these medicinal plants which the current study attempts to provide. From the present study it was observed that the different medicinal plants showed great variations in their onset of flowering and fruiting which were probably due to natural variations among individuals as well as influenced by environmental factors. These two events were observed to sometimes overlap in some of the species especially in *P. angolensis*, *R. vomitoria* *M. lucida* which are typical rainforest ecosystem species. In most instances flowering was majorly a dry season event extending to early rains in some of the species in the lowland and derived savanna while coinciding with late dry season in the

Source of fruiting information	Fruiting records in relation to months in Nigeria												Coordinates of the location												
	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A	M	J	J	A	S	O	N	D	
Unibadan campus																								7°23'N 3°56'E	
Eruwa axis																									7°24'N 3°29'E
Benin axis																									6°19'N 5°41'E
Oyo																									7°50'N 3°55'E
Ogbomoso																									8°07'N 4°14'E
Saki																									8°39'N 3°25'E
Ibilo																									7°26'N 6°04'E
Lokoja axis																									7°49'N 6°44'E
Okene-Kabba																									7°31'N 6°14'E
Makurdi																									7°44'N 8°35'E

Figure 20. Fruiting information in *Rauvolfia vomitoria* in Nigeria in relation to periods of the year across the various ecological zones.

northern range of these species. The occurrence and distribution of some typical savannah medicinal plant species in the rain forest zone and perhaps in derived savanna range was evident of climate change and gradual movement of desert southwards.

From the pre-sent study, the general trends observed provided a good guide for germplasm collections, screening and genetic improvement as well as the development of strategies for their domestication and *ex-situ* conservation strategies across the different species range in Nigeria. Various anthropogenic and environmental threats were also observed for the remaining species germplasm ranging from debarking (*K. senegalensis* and *A boonei*), pollard-ding (*M. lucida*) roots excavation (*S.longipedunculata*, and *R. vomitoria*) as well as seasonal fires and periodic droughts (*Z. xantholxyloides*) among other form of threats

Conflict of Interest

The authors have declared that there no conflict of competing interest.

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Full Length Research Paper

Antioxidant capacity of essential oils extracted from *Lippia sidoides*, *Ageratum fastigiatum*, *Ocotea odorifera*, *Mikania glauca* and their major components

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Received 19 January, 2014; Accepted March 7, 2014

Essential oils are biologically important and a large number of studies to evaluate their antioxidant activities have been performed. The aim of this work was to evaluate the antioxidant capacity of the essential oils of *Lippia sidoides*, *Ageratum fastigiatu*, *Ocotea odorifera*, *Mikania glauca* and their major components, using different assays. The essential oil of *L. sidoides* exhibited the highest antioxidant capacity, presenting the lowest IC₅₀ values for all tests. The essential *L. sidoides* oil, along with the compounds γ -terpinene, carvacrol and thymol, exhibited the highest antioxidant capacity in the β -caroteno/linoleic acid assay. The essential *L. sidoides* oil was the only oil that exhibited an IC₅₀ lower than 40 mg ml⁻¹ in the test that evaluates the antioxidant capacity against the formation of reactive species to thiobarbituric acid, with no statistically significant difference from the IC₅₀ values presented by γ -terpinene, carvacrol, thymol, methyleugenol and antioxidant standards, such as butylated hydroxytoluene (BHT) and α -tocopherol. In the inhibition of 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical, the essential oil from *L. sidoides* exhibited an IC₅₀ value of 86.71 mg ml⁻¹, lower than those presented by carvacrol, thymol and the antioxidant standards. By multivariate analyses of Principal component analysis (PCA) and hierarchical cluster analysis (HCA), it was possible to group the antioxidant activities of essential oils with major components contained in the oils.

Key words: *Lippia sidoides*, antioxidants assays, 1,1-diphenyl-2-picryl hydrazyl (DPPH), thiobarbituric acid reactive substances (TBARS), β -carotene/linoleic acid oxidation system carvacrol.

INTRODUCTION

Food rich in oils and fats can be oxidized during their processing and storage, affecting their quality, and consequently, their consumer acceptance. Moreover, this kind of food stuff could offer risks to the consumer's

health due to the toxic effects caused by the continuous and prolonged ingestion of oxidized products and radicals produced in the oxidation processes (Ramalho and Jorge, 2006). Not only our food, but also our body cells

are exposed to the attack by oxidant agents and free radicals. In cases where these oxidant agents are not controlled, damage may occur to membrane lipids, proteins and also nucleic acids, altering cellular programming (Kinsella et al., 1993).

Toxicological studies performed with synthetic antioxidants (used by the food industry) such as butylated hydroxy anisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and the tert-butylhydroquinone (TBHQ) have demonstrated potential carcinogenic effects in animals (Botterweck et al., 2000). Thus, some research has been focused on finding natural products with antioxidant capacity. In this sense, a lot of studies have been assessed in the antioxidant capacity of the essential oils, which are complex mixtures of several compounds, such as hydrocarbons, alcohol, phenolic compounds among others (Aazza et al., 2011). However, the antioxidant assays currently used can be classified into two categories, those based on the hydrogen direct transfer (direct method) and those based on the electrons transfer (indirect methods) (Huang et al., 2005). Considering that the antioxidants may act through different mechanisms, the assessment of such capacity should be performed by several assays (Miguel, 2010).

The species *Lippia sidoides* Cham., popularly known as “alecrim pimenta” is found in Brazilian northeastern region and it has shown several biological activities, which has been making it a potential source of biologically active components (Cartaxo et al., 2010; Lima et al., 2011). Brazilian “sassafrás” (*Ocotea odorifera* (Vell.) Rohwer) is a tree that can be found from Bahia south to Rio Grande do Sul (Lorenzi, 2008). Its stem essential oil is safrole-rich, but only few studies were performed with the essential oil extracted from the leaves. The species *Mikania glauca* Mart. is an herbaceous climber, found in the state of Minas Gerais in gallery forests (Sano et al., 2008). The *Ageratum fastigiatum* (Gardn.) R. M. King et H. Rob., popularly known as “mata-pasto”, is an invasive plant, found in southeastern Brazil, used by some communities as a healing and antimicrobial (Almeida et al., 2004). According to Del-Vechio-Vieira et al. (2009), in its essential oil there is the predominance of sesquiterpene hydrocarbons.

Essential oils of such species have been used in this study, considering the big variety of compounds contained in them, in a way to represent the several classes, such as terpene hydrocarbons, alcohol, phenylpropanoids, phenolic compounds among others. Moreover, there is the essential oil's good yield presented by

such species. In this context, the aim of this work was to assess the antioxidant capacity of the different essential oils and several components of them, through different methods, in a way to correlate the antioxidant capacity of the essential oil with its chemical compounds, as well as with the different methods.

MATERIALS AND METHODS

Chemicals and reagents

All the standards used were P. A; 1,8-cineole (99%), camphor (97%), p-cymene (99%), linalool (98.5%), α -tocopherol (99%), ascorbic acid (99%), BHT (99%) (Acröss organics, New Jersey, USA); methyleugenol (99%), carvacrol (98%), α -pinene (99%), β -pinene (99%), γ -terpinene (85%), (E)-caryophyllene (98.5%), limonene (98%), thymol (99.5%) (Sigma-Aldrich, Louis, Missouri, USA); α -terpineol (99%) (Merck & CO., Whitehouse Station, New Jersey, USA), 1,1-diphenyl-2-picryl hydrazyl (DPPH), 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH), β -carotene, linoleic acid and thiobarbituric acid reagent were acquired from Sigma-Aldrich, Louis, Missouri, USA. The other chemicals and reagents were from Merck & CO., Whitehouse Station, New Jersey, USA.

Plant

The leaves of *L. sidoides* Cham. (Verbenaceae), *A. fastigiatum* (Gardn.) R. M. King et H. Rob. (Asteraceae), *O. odorifera* (Vell.) Rohwer (Lauraceae) and *M. glauca* Mart. (Asteraceae) were collected in October, 2009, in the morning, in Itumirim – Minas Gerais/Brazil.

Extraction and chromatographic analyses of the essential oils

The essential oils were extracted from fresh leaves of the plants, by hydrodistillation for 2 h in a Clevenger-type apparatus. The essential oils were stored at -20°C in the dark prior to analysis. The qualitative analyses of the essential oils were performed by gas chromatography coupled to a mass spectrometer (GC-MS), a Shimadzu G-17A equipped with a mass selective detector model QP5050A (Shimadzu Corporation, Kyoto, Japan) and a DB-5 capillary column (5% phenylmethylsiloxane, 30 m \times 0.25 mm, i.d., film thickness 0.25 μ m; Folsom, CA, USA). The device was operated in the following conditions: temperature of the ions source at 280°C; programming of the column at an initial temperature 50°C for 2 min with an increase of 4°C/min, up to 200°C, after that, 10°C/min up to 300°C, finalizing at temperature 300°C for 10 min; carrier gas helium (1 ml min⁻¹); ionization energy 70 eV and fragments 40 and 550 Da decomposed. A series of standards of linear hydrocarbons was injected in the same conditions of the sample (C₉H₂₀ C₂₆H₅₄). The mass spectra obtained were compared with the library data bank Wiley 229 and the retention index. In calculating each component, it was compared with the

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one in the table (Adams, 2007).

The quantitative analyses were carried out using a gas chromatography unit equipped with a flame ionization detector (FID), using a Shimadzu CG – 17A device, in the following experimental conditions: DB5 (J&W Scientific Inc., Rancho Cordova, CA, USA), 0.25 mm i.d. × 30 m, 0.25 micron film thickness; temperature of the injector: 250°C; temperature of the FID: 280°C; programming of the column: initial temperature at 60°C for two minutes, followed by heat rate of 4°C/min up to 200°C, after that, another heat rate of 10°C/min up to 300°C, under this temperature for 10 min; carrier gas nitrogen (2.2 ml min⁻¹).

Antioxidant activities of the essential oils and their major components

For each of the three assays we used the IC₅₀ value (concentration that shows 50% of antioxidant capacity) of the essential oils; their major components and antioxidant compounds used as reference were calculated by regression analysis. However, they were calculated only for the essential oils or compounds that show capacity superior to 50% in the concentrations used in each methodology.

β-carotene/linoleic acid oxidation system.

The evaluation of the antioxidant capacity using the β-carotene bleaching was carried out according to the methodologies present by Wang (2008) and Lopes-Lutz et al. (2008) with some slight changes. A solution of β-carotene in chloroform was prepared (2.0 mg ml⁻¹); after that, 60 μl linoleic acid, 600 mg Tween 20® and 1.5 ml chloroform were added to it, and the chloroform evaporated in a rotary evaporator (Büchi R 114 Büchi Corporation, New Castle, USA).

Afterwards, 150 ml distilled water saturated with oxygen was added in the mixture under constant shaking (emulsion A). Moreover, 2.8 ml of emulsion A were added in test tubes and on it 200 μl of the methanolic solutions of the compounds being studied in the concentrations of 5.0, 10.0, 25.0, 50.0, 100.0 and 200.0 μg ml⁻¹ were added. Parallel, two solutions were prepared, one of them without the antioxidant (control) and the other one with the same reagents of A without β-carotene (emulsion B – white). The reading of the samples were performed at 0 and 60 min after adding the antioxidant on emulsion A in a spectrophotometer (Shimadzu UV-160 1PC, Shimadzu Corporation, Kyoto, Japan), at 470 nm. The percentage of inhibition (percentage of antioxidant capacity AA%) was calculated through the following formula: AA% = 100 × (DC – DA) / DC, in which AA (antioxidant capacity); DC (degradation level of the control = [ln (a / b) / 60]); DA (degradation level in the presence of the sample = [ln (a / b) / 60]); a (absorbance at time 0); b (final absorbance, 60 min after the incubation). The antioxidants ascorbic acid, α-tocopherol and BHT were used in the concentrations 5.0, 10.0, 25.0, 50.0, 100.0 and 200.0 μg ml⁻¹.

Formation of reactive species to the thiobarbituric acid (TBARS)

The methodology used by Kulisic et al. (2004) was applied and just some slight changes were made. Initially, in a test tube, 0.5 ml homogenized egg yolk in water (10% p/v) (as lipids source), 0.1 ml methanolic solutions of essential oils and their major components were added and they were previously prepared in the concentrations 5.0, 10.0, 25.0 and 50.0 g L⁻¹. Afterwards, 0.4 ml distilled

water was added, completing the volume in the tube for 1.0 ml. Later, 0.05 ml solutions of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) were added in the concentration 0.07 mol L⁻¹ in water (to induce the lipid peroxidation), 1.5 ml of an acetic acid solution 20% in water (pH 3.5) and 1.5 ml thiobarbituric acid 0.8% p/v diluted in a solution of sodium dodecylsulfate 1.1% p/v in water. After shaking for 30 s, the resulting solution was heated at 95°C for 60 min. It was cooled at room temperature and later, 5.0 ml butan-1-ol was added to each tube, centrifuging the solutions at 1,200 g for 10 min. The organic phase was collected and its absorbance was read by spectrophotometer (Shimadzu UV-160 1PC, Shimadzu Corporation, Kyoto, Japan) at 532 nm. The antioxidant capacity was determined in percentage (AA%), according to the following equation: AA% = [1 - (A_{sample} / A_{control})] × 100, in which A_{Control} are the absorbance values of the solutions without the samples (solutions totally oxidised) and A_{sample} are the absorbance values of the solutions in the presence of the compounds evaluated. In this assay, the antioxidants used as reference, ascorbic acid, α-tocopherol and BHT were used in the concentrations 0.1, 0.4, 2.0 and 4.0 g L⁻¹.

Reduction of the 1,1-diphenyl-2-picryl hydrazyl (DPPH)

The evaluation of antioxidant capacity toward the consumption of the DPPH was realized according to the methodologies of Lopes-Lutz et al. (2008) followed by slight changes. A solution in methanol of DPPH was prepared in the concentration of 40 μg ml⁻¹, which was kept on cooling and under light. In parallel, methanolic solutions of the essential oils and their major components were prepared in the concentrations of 5.0, 10.0, 25.0, 50.0, 100.0 and 500.0 μg ml⁻¹. The reaction mixtures were prepared in test tubes adding 0.3 ml sample solutions at 2.7 ml of DPPH solution in methanol (40 μg ml⁻¹). After that, the readings of absorbance were performed by spectrophotometer (Shimadzu UV-160 1PC, Shimadzu Corporation, Kyoto, Japan) at 517 nm in 60 min after adding both solutions. The percentage of the antioxidant capacity was calculated according to the following equation: [1 - (A_{sample} / A_{control})] × 100, in which A_{sample} was the absorbance of the solution containing all the reagents and A_{control} was the absorbance of solution-control (solution containing all reagents but the compounds evaluated). As a white sample, the mixture (2.7 ml) of methanol and the methanolic solution of the compounds evaluated were used in the respective concentrations. The antioxidant concentrations of ascorbic acid, α-tocopherol and BHT were 1.0, 2.5, 5.0, 10.0, 25.0 and 50.0 μg ml⁻¹.

Statistical analysis

The experimental design was completely randomized with three repetitions. The analyses of variance were performed for the factorial experiments using Scott-Knott test (5%) for means comparison. The analyses were performed by using Sisvar program. The average IC₅₀ values obtained by the regression analyses for all the compounds (essential oils, standards and reference compounds) were compared by Scott-Knott test (5%), using Genes program. The multivariate analysis was also performed using R program. Due to the large number of variables, the analysis of the major components (PCA) and the hierarchical cluster analysis (HCA) were used to analyze the similarity among the essential oils, the standards of the majority components and synthetic antioxidant in relation to IC₅₀ values toward the analysis methods DPPH, β-carotene/linoleic acid and TBARS.

Table 1. Chemical components of the essential oils and respective contents expressed in percentage^a.

Constituent	RI ^b	<i>Lippia sidoides</i>	<i>Ageratum fastigiatum</i>	<i>Ocotea odorifera</i>	<i>Mikania glauca</i>
α-Pinene	924	- ^c	52.79±5.51	0.72±0.04	27.12±0.33
Sabinene	968	3.57±0.25	1.26±0.14	^d Nd	1.82±0.07
β-Pinene	973	0.38±0.03	2.47±0.23	0.56±0.03	21.88±0.33
Myrcene	987	1.70±1.05	0.68±0.05	Nd	23.80±0.23
p-Cymene	1025	9.89±0.31	-	Nd	-
Limonene	1029	Nd	8.41±0.90	0.89±0.02	-
1,8-Cineole	1033	22.63±0.40	-	2.25±0.14	-
γ-Terpinene	1058	7.27±0.11	-	-	-
Linalool	1099	-	-	0.26±0.03	-
Camphor	1148	-	-	7.93±0.35	-
α-Terpineol	1195	0.82±0.04	-	0.32±0.01	-
Thymol	1290	1.17±0.10	-	-	-
Safrole	1293	-	-	12.26±0.77	-
Carvacrol	1302	26.44±0.65	-	-	-
Methyleugenol	1412	-	-	74.03±0.99	-
(E)-Caryophyllene	1419	2.57±0.07	5.48±0.67	-	8.55±0.21
α-Humulene	1455	1.78±0.05	3.87±0.67	-	0.90±0.03
Germacrene D	1483	-	12.26±2.41	-	1.10±0.03
Bicyclogermacrene	1496	-	2.32±0.58	0.27±0.01	8.37±0.30
Caryophyllene oxide	1582	1.54±0.05	Nd	-	0.48±0.02
Junenol	1624	-	3.35±1.38	-	-

^aAverages of three independent extraction CI = $\bar{X} \pm ts/\sqrt{n}$ (n = 5,95% confidence); ^bCalculated Retention index; ^cA dash (-) indicate not detected; ^dNd indicate non-quantified (values < 0.2).

RESULTS

The chemical components of essential oils of *L. sidoides* Cham., *A. fastigiatum* (Gardn.) R. M. King et H. Rob., *O. odorifera* (Vell.) Rohwer and *M. glauca* Mart. followed by their calculated retention index and reported retention index with their contents expressed in percentage (calculated by area normalization) are in Table 1. The values of antioxidant capacity of the essential oils and their major components in relation to their concentrations, evaluated through the assay of β-carotene/linoleic acid oxidation system, were displayed in Table 2. It was observed that all essential oils showed significant antioxidant activities. Among the evaluated compounds, those that belong to the class of phenolic monoterpenes (thymol and carvacrol), phenylpropanoids and sesquiterpenes were the ones that displayed higher activities when taking part in the same concentrations, compared to those presented by the essential oils.

Toward the assay that evaluates the antioxidant capacity by formation inhibition of reactive species to the thiobarbituric acid, the essential oils when presented in the same concentrations, showed lower antioxidant

activities than those showed by phenolic monoterpenes, higher than those of monoterpenes, except γ-terpinene and similar to those of sesquiterpenes, as it can be observed in Table 3.

The assay that assesses the antioxidant capacity according to the reduction of the steady radical DPPH was the most selective one, once only the essential oil of *L. sidoides* and phenolic monoterpenes, carvacrol and thymol showed significant antioxidant activities toward this methodology as in the Table 4. Comparing the IC₅₀ values, it is observed that of the three assays used, the essential oil of *L. sidoides* was the one that showed higher antioxidant capacity, with the lowest IC₅₀ values in all the assays. In the assay of β-carotene/linoleic acid, the essential oil of *L. sidoides* had the highest antioxidant capacity together with the compounds γ-terpinene, carvacrol and thymol. In the assay that evaluates the antioxidant capacity toward the formation of reactive species to the thiobarbituric acid, the only essential oil that had its IC₅₀ value lower than 40 g L⁻¹ was the essential oil of *L. sidoides*, with IC₅₀ equal to 14.27 g L⁻¹. However, this value did not differ statistically from the other IC₅₀ values given by γ-terpinene, carvacrol, thymol,

Table 2. Values of antioxidant capacity of the essential oils and their major components expressed in percentage of inhibition toward the assay β -carotene/linoleic acid.

Compound	Concentration ($\mu\text{g ml}^{-1}$) ^a						IC ₅₀ ($\mu\text{g ml}^{-1}$)
	5	10	25	50	100	200	
Essential Oils							
<i>Ocotea odorifera</i>	15.82 ^{aC}	23.51 ^{bC}	37.74 ^{cC}	40.91 ^{cD}	61.82 ^{dG}	72.84 ^{eE}	64.97 ^D
<i>Lippia sidoides</i>	60.08 ^{aE}	75.05 ^{bG}	84.56 ^{cF}	87.80 ^{cG}	90.20 ^{cI}	88.49 ^{cF}	4.23 ^H
<i>Mikania glauca</i>	2.38 ^{aA}	6.38 ^{aB}	20.37 ^{bB}	31.08 ^{cC}	43.20 ^{dE}	59.07 ^{eC}	111.85 ^A
<i>Ageratum fastigiatum</i>	17.05 ^{aB}	19.36 ^{aD}	34.81 ^{bC}	46.69 ^{cD}	50.16 ^{cF}	66.26 ^{dD}	79.21 ^B
α -Pinene	1.74 ^{aA}	3.23 ^{aA}	7.35 ^{aA}	16.12 ^{bB}	48.52 ^{cF}	71.27 ^{dE}	116.79 ^A
β -Pinene	1.29 ^{aA}	1.69 ^{aA}	9.53 ^{bA}	15.75 ^{bB}	30.37 ^{cC}	47.44 ^{dB}	- ^b
ρ -Cymene	6.43 ^{aA}	5.97 ^{aB}	12.33 ^{bA}	16.84 ^{bB}	18.43 ^{bA}	19.01 ^{bA}	-
Limonene	13.41 ^{aB}	10.61 ^{Ab}	18.54 ^{bB}	21.48 ^{bB}	32.35 ^{cC}	38.79 ^{cB}	-
1,8-Cineole	4.22 ^{aA}	13.44 ^{bC}	13.72 ^{bB}	14.29 ^{bA}	14.55 ^{bA}	29.90 ^{cA}	-
Camphor	3.31 ^{aA}	2.69 ^{aA}	19.22 ^{bB}	20.16 ^{bB}	23.70 ^{bB}	41.53 ^{cB}	-
γ -Terpinene	28.80 ^{aC}	57.33 ^{bF}	68.82 ^{cE}	78.28 ^{dF}	82.01 ^{dH}	87.50 ^{eF}	11.25 ^F
α -Terpineol	1.46 ^{aA}	2.15 ^{aB}	7.99 ^{aA}	7.27 ^{aA}	13.76 ^{bA}	13.98 ^{bA}	-
Linalool	0.25 ^{aA}	1.33 ^{aA}	11.94 ^{bA}	21.38 ^{cB}	36.41 ^{dD}	41.38 ^{dB}	-
Safrole	-4.62 ^{aA}	3.92 ^{bA}	32.47 ^{cC}	61.21 ^{dE}	63.89 ^{dG}	75.64 ^{eE}	66.86 ^D
Carvacrol	65.67 ^{aE}	76.01 ^{bG}	84.17 ^{cF}	86.64 ^{cG}	89.29 ^{cI}	92.20 ^{cF}	3.85 ^H
Thymol	36.05 ^{aD}	49.56 ^{bE}	82.25 ^{cF}	87.44 ^{dG}	89.43 ^{dI}	90.71 ^{dF}	6.76 ^G
Methyleugenol	2.56 ^{aA}	9.24 ^{aA}	22.72 ^{bB}	35.95 ^{cC}	50.10 ^{dF}	69.12 ^{eE}	82.41 ^B
(E)-Caryophyllene	17.21 ^{aB}	22.22 ^{aD}	46.84 ^{bD}	67.11 ^{cE}	80.15 ^{dH}	86.34 ^{dF}	36.27 ^E
Standards	-	-	-	-	-	-	-
Ascorbic acid	-	-	-	-	-	-	71.43 ^C
α -Tocopherol	-	-	-	-	-	-	<0.15
BHT	-	-	-	-	-	-	<0.15

^aMeans followed by the same lower case in the same line and by the same capital letter in the same column do not differ significantly by Scott-Knott Test ($p = 0.05$); ^bA dash (-) indicate compounds that show IC₅₀ values higher than 200 $\mu\text{g ml}^{-1}$.

Table 3. Values of antioxidant capacity of the essential oils and their major components in percentage of inhibition using TBARS method.

Compound	Concentration (g L^{-1}) ^a				IC ₅₀ (g L^{-1})
	5	10	25	50	
Essential oils					
<i>Ocotea odorifera</i>	11.38 ^{aD}	22.71 ^{bE}	25.53 ^{bD}	45.55 ^{cE}	- ^b
<i>Lippia sidoides</i>	18.36 ^{aE}	37.10 ^{bF}	54.29 ^{cF}	54.12 ^{cF}	14.27 ^B
<i>Mikania glauca</i>	12.50 ^{aD}	33.52 ^{bF}	34.74 ^{bE}	46.95 ^{cE}	-
<i>Ageratum fastigiatum</i>	6.88 ^{aC}	13.69 ^{bD}	24.46 ^{cD}	26.56 ^{cD}	-
α -Pinene	3.90 ^{aB}	4.04 ^{aC}	5.78 ^{aB}	6.27 ^{aB}	-
β -Pinene	9.70 ^{aD}	11.80 ^{aD}	21.12 ^{bD}	27.05 ^{cD}	-
ρ -Cymene	-1.09 ^{aA}	-0.44 ^{aB}	1.08 ^{aA}	0.75 ^{aA}	-
Limonene	-2.09 ^{aA}	1.95 ^{aC}	14.54 ^{bC}	18.35 ^{bC}	-
1,8-Cineole	-1.57 ^{aA}	-5.96 ^{aA}	-0.96 ^{aA}	-0.41 ^{aA}	-
Camphor	4.54 ^{aB}	7.56 ^{aC}	13.82 ^{bC}	14.21 ^{bC}	-
γ -Terpinene	24.22 ^{aF}	41.07 ^{bG}	72.68 ^{cH}	84.21 ^{dI}	8.51 ^C
α -Terpineol	3.84 ^{aB}	14.46 ^{bD}	34.83 ^{cE}	45.30 ^{dE}	-
Linalool	0.52 ^{aA}	3.51 ^{aC}	16.74 ^{bC}	17.44 ^{bC}	-
Safrole	9.51 ^{aD}	11.95 ^{aD}	29.69 ^{bE}	45.76 ^{cE}	-
Carvacrol	37.82 ^{aH}	61.03 ^{bH}	71.53 ^{cH}	77.27 ^{dH}	3.25 ^C
Thymol	31.61 ^{aG}	44.38 ^{bG}	63.07 ^{cG}	71.84 ^{dG}	9.21 ^C

Table 3. Contd.

Methyleugenol	1.15 ^{aA}	16.26 ^{bD}	32.27 ^{cE}	50.73 ^{dF}	39.20 ^A
(E)-Caryophyllene	5.53 ^{aC}	6.98 ^{aC}	21.55 ^{bD}	29.04 ^{cD}	-
Standards	-	-	-	-	-
Ascorbic acid	-	-	-	-	2.43 ^C
α-Tocopherol	-	-	-	-	0.78 ^C
BHT	-	-	-	-	2.65 ^C

^aMeans followed by the same lower case in the same line and by the same capital letter in the same column do not differ significantly by Scott-Knott Test ($p = 0.05$); ^bA dash (-) indicate compounds that show IC₅₀ values higher than 40 g L⁻¹.

Table 4. Antioxidant capacity of the essential oils and their major components expressed in percentage of inhibition, using the reduction method of DPPH.

Compound	Concentration (µg mL ⁻¹) ^a						IC ₅₀ (µg mL ⁻¹)
	5	10	25	50	100	500	
Essential oils							
<i>Ocotea odorifera</i>	0.02 ^{aA}	0.06 ^{aA}	0.66 ^{aA}	2.61 ^{bB}	2.87 ^{bB}	8.59 ^{cC}	- ^b
<i>Lippia sidoides</i>	5.42 ^{aB}	11.91 ^{bB}	23.57 ^{cB}	37.35 ^{dC}	52.30 ^{eD}	84.33 ^{fF}	86.71 ^A
<i>Mikania glauca</i>	0.38 ^{aA}	0.13 ^{aA}	0.32 ^{aA}	0.82 ^{aA}	0.13 ^{aA}	1.01 ^{aA}	-
<i>Ageratum fastigiatum</i>	0.56 ^{aA}	0.57 ^{aA}	0.76 ^{aA}	0.85 ^{aA}	0.85 ^{aA}	3.06 ^{bB}	-
α-Pinene	0.13 ^{aA}	0.20 ^{aA}	0.30 ^{aA}	0.53 ^{aA}	0.57 ^{aA}	0.75 ^{aA}	-
β-Pinene	0.31 ^{aA}	0.31 ^{aA}	0.35 ^{aA}	0.35 ^{aA}	0.54 ^{aA}	1.46 ^{aB}	-
p-Cymene	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.03 ^{aA}	-
Limonene	0.00 ^{aA}	0.03 ^{aA}	0.09 ^{aA}	0.29 ^{aA}	0.33 ^{aA}	0.33 ^{aA}	-
1,8-Cineole	0.25 ^{aA}	0.00 ^{aA}	0.13 ^{aA}	0.17 ^{aA}	0.37 ^{aA}	0.37 ^{aA}	-
Camphor	0.03 ^{aA}	0.10 ^{aA}	0.23 ^{aA}	0.36 ^{aA}	0.36 ^{aA}	0.50 ^{aA}	-
γ-Terpinene	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	-
α-Terpineol	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	-
Linalool	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.64 ^{aA}	-
Safrole	0.01 ^{aA}	0.11 ^{aA}	1.31 ^{bA}	2.32 ^{bB}	4.77 ^{cC}	22.58 ^{dD}	-
Carvacrol	10.63 ^{aC}	18.15 ^{bC}	31.84 ^{cC}	44.51 ^{dD}	58.30 ^{eE}	83.83 ^{fF}	71.95 ^B
Thymol	20.73 ^{ad}	27.26 ^{bD}	39.64 ^{cD}	49.91 ^{dE}	61.82 ^{eF}	84.88 ^{fF}	59.85 ^C
Methyleugenol	0.16 ^{aA}	0.81 ^{aa}	0.91 ^{aA}	2.01 ^{bB}	4.62 ^{cC}	26.98 ^{dE}	-
(E)-Caryophyllene	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.17 ^{aA}	0.47 ^{aA}	-
Standards	-	-	-	-	-	-	-
Ascorbic acid	-	-	-	-	-	-	4.43 ^E
α-Tocopherol	-	-	-	-	-	-	8.47 ^D
BHT	-	-	-	-	-	-	7.57 ^D

^aMeans followed by the same lower case in the same line and by the same capital letter in the same column do not differ significantly by Scott-Knott Test ($p = 0.05$); ^bA dash (-) indicate compounds that show IC₅₀ values higher than 500 µg mL⁻¹.

methyleugenol and the antioxidant standards. In the reduction assay of the DPPH, IC₅₀ value for this essential oil was 86.71 µg mL⁻¹, showing the lowest capacity of this essential oil in relation with the activities presented by carvacrol, thymol and antioxidant standards.

The PCA showed that with the first and second major components, it was possible to describe 100% of the data, being 98.9% of the total variance described by the

first major component. The analysis of IC₅₀ values, by PCA technique, for the antioxidant activities of the essential oils, the standards of the majority of components and the synthetic antioxidants by the three assays, permitted us to cluster the samples in four groups in order to express and evidence their similarities and differences (Figure 1A).

The analysis of the biplot graphic PC1 × PC2 of the

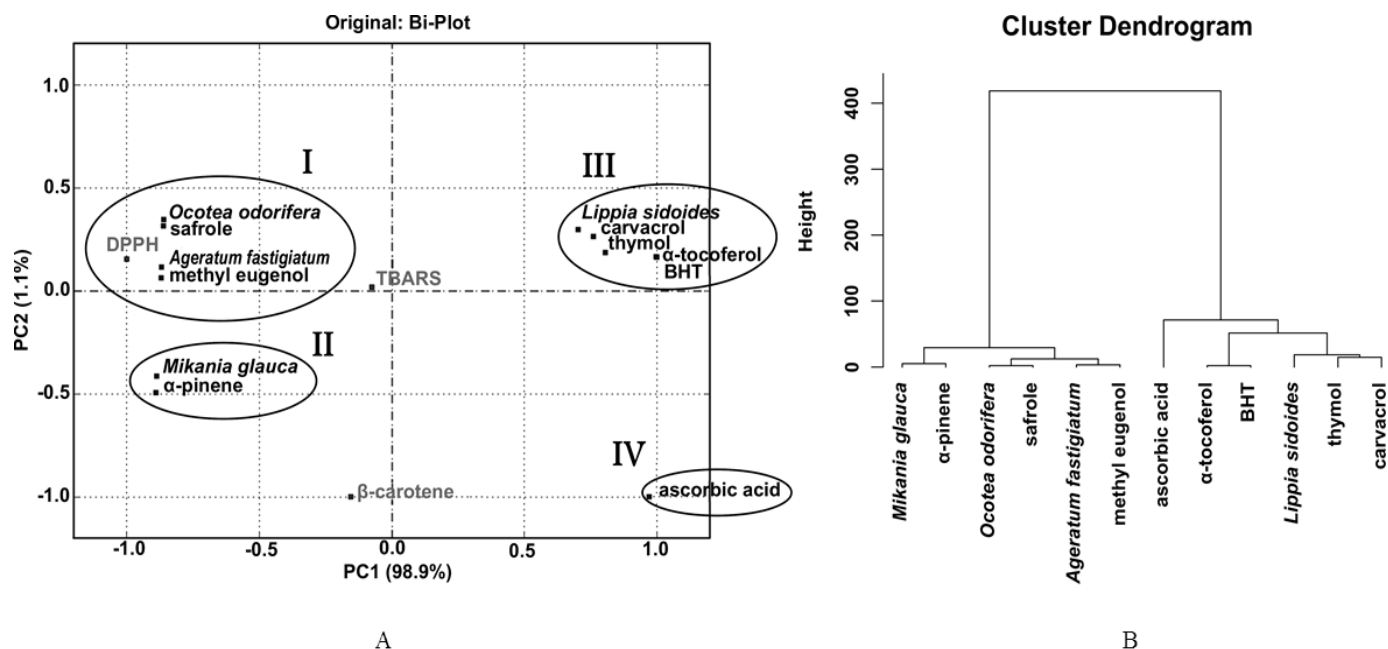


Figure 1. A) Biplot graphic PC1 × PC2 of the loadings and scores for IC_{50} and B) Dendrogram of IC_{50} values of different essential oils, standards of the major components and synthetic antioxidants in relation to the three assays used.

'loadings' and 'scores' shows, by a bi-dimensional graphic, the split of the samples in four groups, specifically, group I (*O. odorifera*, *A. fastigiatum*, safrole and methyleugenol), group II (*M. glauca* and α -pinene), group III (*L. sidoides*, carvacrol, thymol, BHT and α -tocopherol) and group IV (Ascorbic acid). The dendrogram (Figure 1B) of IC_{50} values, for the antioxidant activities of the samples, corroborated with the result of the major components analysis, highlighting the existent cluster among *O. odorifera*, *A. Ageratum*, safrole and methyleugenol; *M. glauca* and α -pinene; *L. sidoides*, carvacrol, thymol, BHT and α -tocopherol; and ascorbic acid. This means IC_{50} values for the antioxidant activities of the essential oils behaved similarly with their majority components, except the essential oil of *A. Ageratum*.

DISCUSSION

According to these results, it was possible to claim that the essential oils rich in terpenes had better values for antioxidant capacity for the oxidant assay of β -carotene/linoleic acid system. Similar results were found by Hussain et al. (2008), who evaluated the antioxidant capacity of the essential oil of *O. basilicum* by the methodologies of β -carotene/linoleic acid and DPPH. The results found in the reduction assay of DPPH demonstrated

the existence of antioxidant capacity only for the compounds that presented a high capacity of donating an electron as phenolic compounds. Corroborating with the antioxidant activities showed the essential oils of *O. basilicum*, *Origanum vulgare* and *Thymus vulgaris*, rich in methyl chavicol (estragole) (45.8%), carvacrol (61.3%) and thymol (47.9%), respectively (Bozin et al., 2006). Regarding the methodology evaluating the antioxidant capacity by the inhibition of the formation of reactive species to the thiobarbituric acid (TBARS), all the essential oils can be considered antioxidant (Table 3). However, the essential oil that showed higher capacity was *L. sidoides* (54.29%), followed by the oils of *O. odorifera* (45.55%), *M. glauca* (46.95%) and *A. Ageratum* (26.56%). The only essential oil that showed IC_{50} value lower than 50 g L^{-1} was the essential oil of *L. sidoides*, which had IC_{50} value statistically equal to the values displayed by the compounds, thymol, carvacrol and γ -terpinene and the antioxidants ascorbic acid, BHT and α -tocopherol (Table 4). These results corroborated with those described by Kulisic et al. (2004) which did not find differences between the antioxidant capacity showed by the essential oil of *O. vulgare* and their fractions rich in compounds that had the groups CHO and CH and their structures, such as the compounds thymol and carvacrol.

The high antioxidant capacity presented by γ -terpinene toward TBARS assay is according to the results found by

Ruberto and Baratta (2000) who, using the same methodology, evaluated the antioxidant capacity of almost 100 pure compounds that were generally found in several essential oils.

Conclusion

The essential oils rich in terpenes showed higher antioxidant capacity toward the oxidation assay of β -carotene/linoleic acid system while those that had phenolic compounds as major components showed high antioxidants activities toward this assay and also the reduction assays of DPPH and formation of reactive species to the thiobarbituric acid.

ACKNOWLEDGMENTS

The authors thank Minas Gerais State Research Foundation (FAPEMIG) and the National Council for Scientific and Technological Development (CNPq) for their financial support.

CONFLICT OF INTEREST

The authors have declared that there no conflict of competing interest.

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