In Vitro Studies on Antioxidant Properties of Gallic Acid from Mangifera Indica

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Polyphenolic compounds exert a variety of physiological effects in vitro and are able to act as antioxidants by virtue of their hydrogen-donating and metal-chelating capacities. Gallic acid from Mangiferaindica kernel was isolated and identified. This article describes an approach to the study of the antioxidant activity of gallic acid. This activity is compared with a known antioxidant quercetin. The 1, 1-diphenyl-2-picryhydarzyl (DPPH), superoxide & hydroxyl radical scavenging activities and inhibition of ferrous sulphate induced lipid peroxidation of this compound was evaluated to determine its physiological usefulness as protective against oxidative injury. Gallic acid mainly exhibited a potent scavenging effect on superoxide and 1, 1-diphenyl-2-picryhydarzyl (DPPH) radicals and also acted as a moderate scavenger of hydroxyl radicals. The antioxidative protection of low-density lipoprotein (LDL) was also evaluated and compared with that of quercetin, because the generation of oxidized LDL is one of the most active and specific risk factors contributing to atherogenesis.

Keywords**:** Hydroxyl Radical, Antioxidant, Gallic Acid and Serum Oxidation.

Introduction

Flavonoids are polyphenolic components of higher plants known to be excellent antioxidants *in vitro*. Dietary flavonoids have been shown to prevent lipid peroxidation, to scavenge reactive oxygen species, to chelate iron ions, essential for the generation of hydroxyl radicals, and to inhibit NADPH-dependent oxidases and consequently superoxide anion production (1; 2; 3). Evidence for the potential role of oxidative stress in various diseases and pathophysiological processes suggests that the dietary intake and the therapeutic use of flavonoids may have positive health effects (4; 5; 6).

Polyphenols are reducing agents, and together with other dietary reducing agents, such as vitamin C, vitamin E and carotenoids, referred to as antioxidants, protect the body's tissues against oxidative stress and associated pathologies such as cancers, coronary heart disease and inflammation (2; 7). The variable response to dietary flavonoids could have important physiological consequences since individual flavonoids and their metabolites have differing biological effects (8). A significant body of literature has accumulated, primarily from *in vitro* investigations, regarding the antioxidant properties of flavonoids and other plant polyphenolics (9). In recent years improved understanding of the pharmacological properties of individual flavonoid compounds has led to the developments of flavonoid drugs.

Many fruits and vegetables have already been identified as good antioxidants due to the presence of good quality antioxidants like flavonoids and other polyphenolic compounds in addition to usual antioxidants like ascorbic acid, α- tocopherol, β- carotene etc. (10). However, supplementation of these antioxidants through diet does not always meet the requirements. Effective drugs developed from natural sources come to rescue under such situations. Since 'stress' as well as 'pollution' induces the generation of toxic radicals in the body and modern man is always under the purview of these risk factors, antioxidants from diet alone may not compensate the issue. Accumulation of toxic radicals over periods can cause life threatening diseases like cardiovascular diseases and cancer. Hence it is highly demanding to unmask the best antioxidant hidden in natural sources.

Materials and Methods

Part A: Isolation and characterization

The mango kernel was air dried and the ground dried material was extracted with hot 80% methanol thrice (Petra *et al*, 1999). The combined extract was evaporated to dryness and the residue was dissolved in water and extracted successively with hexane, benzene, ethyl acetate and n-butanol. The respective extracts were evaporated in vacuum yielding residues from hexane, benzene, ethyl acetate, and n-butanol. Ethyl acetate residue was selected for column chromatography because of its high polyphenolic content. Ethyl acetate residue was used for successive column chromatography with eluents such as hexane, chloroform, ethyl acetate, and methanol mixtures in increasing polarity and repeated column chromatography of series ($CHCl_3$: ethyl acetate, 1: 9) afforded the compound, which was examined further. Yield of compound was 335.2 mg/Kg.

The compound that has been obtained by column chromatographic separation and subsequent crystallization from ethanol appears to be slightly yellow colored crystals, which is soluble in methanol, ethanol and other organic solvents. It was also soluble in dilute sodium bicarbonate solution and gave indication of effervescence. This pointed to an acidic nature of the compound. For its structure identification, it was subjected to spectral analysis. Its UV-visible spectrum showed a λ max at 269 nm and another at λ 396 nm. In presence of sodium hydroxide its absorption spectrum underwent a drastic change thus indicating a phenolic nature for the compound.

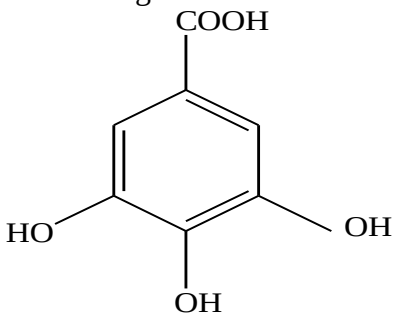
Its UV-visible spectrum showed a shift in the λ max in the presence of $AlCl_3$ (the shift observed was +44 nm) and $AlCl_3$ +HCl (the shift observed was + 12 nm), which is characteristics of the presence of orthodihydroxy groups. These UV- visible spectral parameters points to the fact that the compound presently isolated could be a phenolic acid. Its IR spectrum in KBr disc showed a broad band in the region 2600- 3350 cm^{-1}. This seemingly indicates the presence of OH groups in the molecule.

There was a strong peak at 1700 cm^{-1}, which is attributed to a carbonyl function. The ^1H NMR spectrum of the compound was taken in methanol, acetone and DMSO-d_6. These spectra showed the absence of any methoxy, alkyl or alkene functionalities. There were only aryl hydrogens seen in the spectra. The ^{13}C NMR spectrum also was in accordance with the above

conclusion; peaks appeared only in the range 95- 167 ppm. These were at 95.73, 109.0, 120.74, 137.78, 145.18, and 167.76 ppm and are indicative of an aromatic ring bearing oxygen function. The ES- MS showed a peak at m/z 188.

The ES- MS was run in presence of ammonium acetate in methanol and therefore the peak at m/z 188 is assigned to [M + NH_4] thereby giving 170 as the molecular mass of the compound under investigation. This is substantiated by the appearance of a peak at m/z 358 which is assignable to [2 M+ NH_4^+] peak. Such cluster peaks are the hallmark of electrospray mass spectrum. In addition, similar [n M + NH_4^+] clusters were seen at n values of 3, 4, 5, and 6 at m/z values of 528, 698, 868 and 1038, thus confirming that the molecular mass is 170.

Considering all the data above, it appears that the compound has a carbonyl, at least two hydroxyls and a benzene ring. This leads to a plausible structure of [2HO + C_6H_3 + COOH] with mass 154. If another oxygen is present as in $C_7H_6O_5$, then the molecule could be a trihydroxy aromatic carboxylic acid. The singlet nature of the ^{1}H NMR peak at 7.06 indicates that the compound could be 3, 4, 5- trihydroxy benzoic acid or gallic acid. The melting point reported for gallic acid is 251°C; the compound presently isolated melts at 251 °C. Thus, the compound isolated could be conclusively identified as gallic acid.



3, 4, 5- trihydroxy benzoic acid or gallic acid

PART B: In vitro studies on antioxidant activities of gallic acid from Mangiferaindica kernel

Flavonoids and other polyphenolic compounds are the most potent antioxidants. Polyphenols can form complexes with reactive metals such as iron, zinc and copper- reducing their absorption. At first glance, this may seem to be a negative side effect (reducing nutrient absorption), but excess levels of such elements (metal cations) in the body can promote the generation of free radicals and contribute to the oxidative damage of cell membranes and cellular DNA (Sestili *et al*, 2002). In addition to their chelating effect on metal cations, polyphenols also function as potent free radicals before they can cause cellular damage (Bravo, 1998; Damianaki *et al*, 2000; Fuhrman *et al*, 1995; Goldbohm *et al*, 1996; Kuo, 1997). In general, flavonoids and other polyphenolic compounds are thought to deliver health benefits by several mechanisms, including:

- direct free radical quenching
- protection and regeneration of other dietary antioxidants (like vitamin E)
- chelation of metal ions

Both metal chelating and free radical scavenging activities have been recognized as the antioxidant mechanism for flavonoids in a biological system (Afanas'ev *et al*, 1989; Belinky *et al*, 1998). Flavonoids can act as chain breaking antioxidants by scavenging chain propagating peroxyl radicals because they possess phenolic hydrogens responsible for the peroxyl scavenging activity. Bors *et al* (1990) have proposed that three structural groups are important determinants for free radical scavenging; (a) the o- hydroxyl (catechol) structure in the B ring, which is obvious radical target site for all flavonoids, (b) the 2, 3- double bond in conjunction with 4- oxo function, which is responsible for electron delocalization (c) the additional presence of both 3- and 5- hydroxyl groups for maximal radical scavenging potentials and strongest radical absorption.

Numerous in-vitro studies have shown that polyphenolic compounds are powerful antioxidants that can protect cell membranes and cellular DNA from the damaging effects of free radical induced oxidative damage (Sestili *et al*, 2002; Rice- Evans et al, 1997). Here we have used the purified gallic acid from Mangiferaindica for in vitro studies including $FeSO_4$ induced lipid peroxidation (Tripathi and Pandey, 1999), inhibition of superoxide production (Rowley and Halliwell, 1983), antiradical efficiency (Joyeux et al, 1995), effect on serum oxidation (Hodgson et al, 1999) and inhibition on hydroxyl radical formation (Jeffery et al, 1989). The effects of the compound were compared with quercetin, a known antioxidant flavonoid purchased from Sigma Chemical Company, USA.

Statistical Analysis

The data given in tables and figures are the mean of the values from the number of animals specified in the respective tables and figures ± SEM. Statistical significance was determined by One-way Analysis of Variance (ANOVA) in SPSS 10.0 package. Paired comparison between groups was made by Duncan's multiple range test. 'p' value of 0.05 or less was considered as significant. Values expressed as mean ± SEM, for n = 6.

Results

Effect of gallic acid from *Mangiferaindica* on $FeSO_4$ induced lipid peroxidation (fig 1):

The lipid peroxidation was inhibited of by gallic acid from *Mangiferaindica* in concentration dependent manner. Similarly, quercetin also inhibited in concentration dependent manner. Concentration required to produce 50% inhibition on lipid peroxidation (IC_{50}) was 42.5 ± 1.275 μg/ 3ml in the case of gallic acid where as it was 44.63 ± 1.78 μg/ 3ml for quercetin.

Inhibition of superoxide production (fig 2):

The superoxide production was inhibited by gallic acid from *Mangiferaindica* in a concentration dependent manner. Concentration of flavonoid required to induce 50 % (IC_{50}) inhibition is 4.35 ± 0.18 μg/ 3ml for gallic acid whereas 34 ± 1.36 μg/ 3ml for quercetin.

Effect of gallic acid on antiradical activity (fig 3):

Antiradical efficiency also increased with concentration. Concentration of flavonoid required to induce 50 % inhibition (IC_{50}) is 3.83 ± 0.167 μg/ 2ml for gallic acid from *Mangiferaindica* whereas 26.13 ± 1.12 μg/ 2ml for quercetin.

Effect of gallic acid on hydroxyl radical scavenging (fig 4):

Gallic acid from *Mangiferaindica* and quercetin showed 50 % inhibition on hydroxyl radical production at 6.375 ± 0.22 μg/ 2ml, 9.5 ± 0.4 μg/ 2ml and 11.063 ± 0.47 μg/ 2ml respectively.

Effect of gallic acid on serum oxidation (Fig 5):

The lag time to lipoprotein formation was measured from the plot of absorbance against time. The lag time was defined as the intercept between the tangent of the absorbance curve during the propagation phase and baseline. The lag time of compounds to lipoprotein diene formation in serum oxidation are given in table 1.

Discussion

In vitro studies on inhibition of production of superoxide's, and hydroxyl radicals, antiradical efficiency, and serum oxidation showed that gallic acid from *Mangiferaindica* was highly effective antioxidant. Gallic acid from *Mangiferaindica* exerted 50% inhibition (IC_{50}) of superoxide production at a concentration of 4.35 ± 0.18μg whereas quercetin showed a higher value 34 ± 1.36μg. This compound also acted as efficient radical scavenger and inhibition was found to be 50 % at a concentration of 3.83 ± 0.167μg for gallic acid in spite of the fairly high concentration 26.13 ± 1.12μg for quercetin.

This is in accordance with several other reports demonstrating the property of inhibiting autoxidation reactions and scavenging of free radicals by flavonoids (Galati *et al*, 2002). Flavonoids possess multiple properties for scavenging reactive oxygen and nitrogen species (van Acker *et al*, 1995; Rice- Evans, 1999). Flavonoids inhibit platelet activation by interfering simultaneously with several biochemical pathways, as platelets are likely to be exposed *in vivo* to stimulation by several agents acting through different mechanisms (Beretz and Cazenave, 1988). Flavonoids can react with superoxide anions (Afanas'ev *et al*, 1989), hydroxyl radicals (Husain *et al*, 1987), and lipid peroxy radicals (Torel*et al*, 1986). These compounds may also act by chelating iron (Afanas'ev *et al*, 1989; Morel *et al*, 1998) which is thought to catalyze processes leading to the appearance of free radicals.

Kim (2001) explained the antioxidant potential of biflavones of *Ginkgo biloba* on the basis of structure-related activity and hydroxy- and methyl-substitutions on the basic structure of these flavonoids. Flavonoid rich extract from rose hip (*Rosa canina*) inhibited superoxide anions, hypochlorous acid (HOCl) and hydrogen peroxide (H_2O_2) generated by *in vitro* inflammatory conditions induced on isolated PMN (Daels-Rakotoarison *et al*, 2002). It has recently been suggested that phenolic/flavonoid antioxidants from apple extracts inhibited proliferation of tumor cells *in vitro* (Lapidot *et al*, 2002). Plant polyphenols, such as gallic acid, have been reported to have a range of biological activities including antimutagenic effects (Stupans *et al*, 2002).

Gallic acid is a naturally occurring plant phenol (found in green tea & grape seed extract). In screening anti-cancer agents, gallic acid was found to show cytotoxicity against all cancer cells that were examined. Additionally, the study found that gallic acid did not harm healthy cells, but was able to distinguish between normal cells and cancer cells (Inoue *et al*, 1995). A number of antioxidant phenols, pyridines, and gallic acid esters are believed to be effective by virtue of their antioxidant action. A direct relation between radical inhibitory action and radiation protection has been observed (Burlakova *et al*, 1965). The protective effect of gallic acid esters are attributed to inhibition of chain oxidation processes induced by radiation (Hasan *et al*, 1981).

Activity-guided fractionation of the ethyl acetate soluble fraction from *Chrysophyllumcainito* L. (Sapotaceae), known commonly as star apple or caimito, was performed to identify the antioxidant constituents, which contained gallic acid as one of nine polyphenolic antioxidants (Luo *et al*, 2002). Bisignano *et al*, (2000) reported the antibacterial activity of gallic acid isolated from *Mitracarpusscaber*, a species used in folk medicine by West African native people and the minimum inhibitory concentration of gallic acid was found as 3.90 µg /ml for the inhibition of the growth of *Staphylococcus aureus*. A water extract of *Limoniumwrightii* showed a strong scavenging action for the 1, 1-diphenyl-2-picrylhydrazyl, or superoxide anion and moderate for hydroxyl radical. Gallic acid was identified as the active component of *Limoniumwrightii* with a strong free radical scavenging action (Aniya *et al*, 2002). However, growth retardation and toxicity symptoms were assigned to gallic acid when fed to rats at dietary levels of 2-10% (Joslyn and Glick, 1969).

In our highly industrial and technological society, the pharmaceutical industry had been disrupting the ancient relationship between man and plants. However, there is already a decided swing back to the old ways. People are beginning to take a greater interest in herbs and their uses, and grandmother's remedies are coming into their own again. Rediscovery of old truths and

integration of the traditional medical system with new technology can generate wonderful drugs without any side effects.

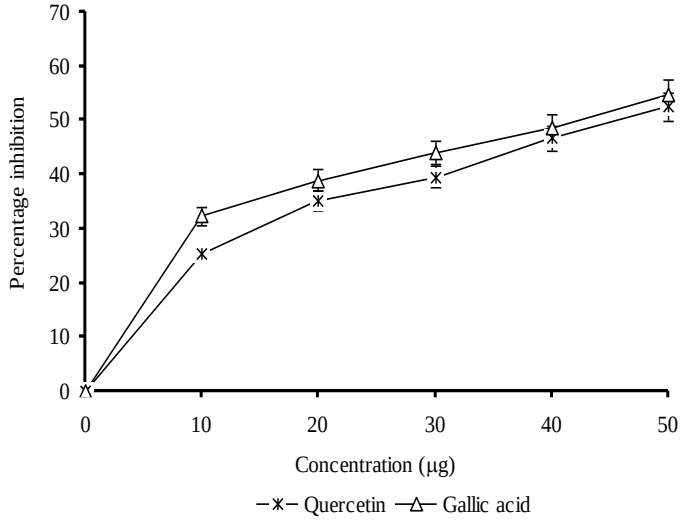


Fig 1. Effect of Gallic Acidon $FeSO_4$ induced Lipid Peroxidation

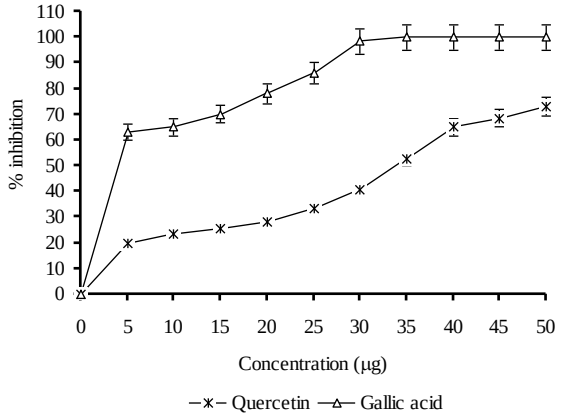


Fig 2. Percentage inhibition of Superoxide Production.
Values expressed as mean ± SEM, for n = 6.

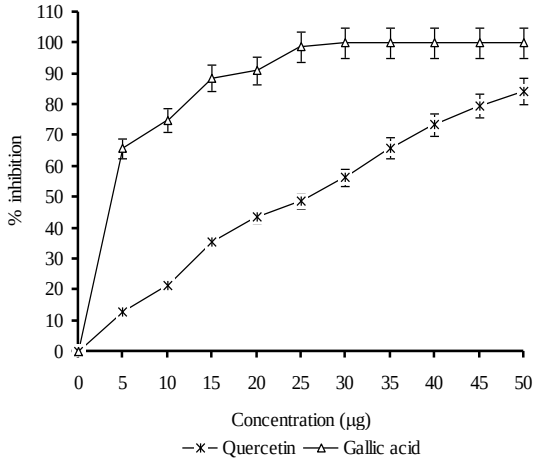


Fig 3. Antiradical Efficiency

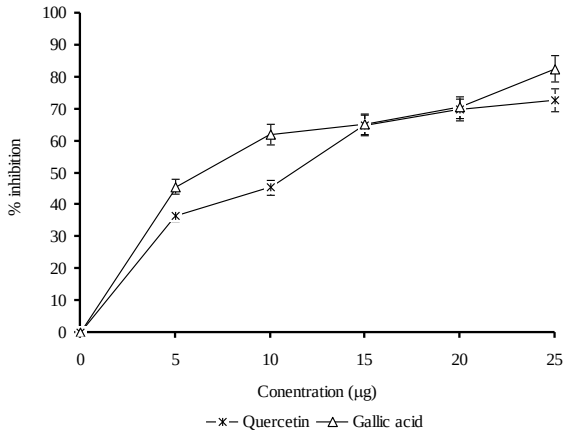


Fig 4. Effect of Gallic Acid on Hydroxyl Radical Scavenging

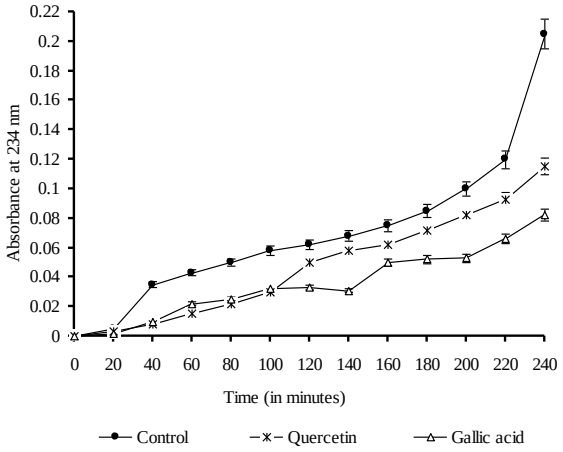


Fig 5. Effect of Gallic Acid on Serum Oxidation

Sample	Lag time (in minutes)
Control	17
Quercetin (50 μg)	71
Gallic acid (50 μg)	108

Table 1. Effect of Gallic Acid on Lag Time for Lipoprotein Diene Formation

Reference

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