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Evaluation of the Antioxidant Activity of the Secondary Metabolites from *Piliostigma reticulatum* (DC.) Hochst

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Abstract: The ethyl acetate extract of *Piliostigma reticulatum* leaves was investigated for its antioxidant constituents. Isolated secondary metabolites of the leaves extract include: 2-O-(*p*-hydroxyphenyl)-5-hydroxy-6-C-methyl-7-methoxychromone (piliostigmin) 1, 6-C-methylkaempferol-3-methyl ether 2, quercetin 3, quercetrin (quercetin-3-O-rhamnose) 4, and Isoquercetrin (quercetin-3-O-glucoside) 5. These compounds were evaluated for their antioxidant potentials. A rapid test for antioxidants using DPPH (1,1-diphenyl-2-picrylhydrazyl) TLC screening, demonstrated considerable radical scavenging activity for all the compounds. The DPPH spectrophotometric method was employed for quantitative determination of radical scavenging activity. The activity was expressed as EC₅₀ value, which ranges between 4.68±0.03 and 50.76±1.71 for the flavonoids constituent.

Key words: *Piliostigma reticulatum*, antioxidant activity, flavonoids, free radicals, DPPH

INTRODUCTION

Recent advances in medical sciences have linked free radicals generated in the body in some diseased conditions. Free radicals have been reported to initiate inflammation, cancer and hypertension^[1] among others. Attention is being shifted to the possibility of using antioxidants to arrest the adverse effect of free radicals^[2]. For this reason, there is an increasing interest in antioxidants to arrest free radicals formation in the body and to prevent incidence of auto-oxidation in foodstuffs

leading to off-flavor development. Antioxidants compounds obtained from natural sources are receiving considerable attention due to the carcinogenicity of the synthetic ones^[3]. Antioxidant activity can be measured in many different ways. In this study, antioxidant activity was determined on isolated secondary metabolites (Fig. 1) using two DPPH methods: TLC rapid screening technique and DPPH spectrophotometric assay.

Piliostigma reticulatum (DC.) Hochst (family: Caesalpinaceae) is an African medicinal plant, widely used in the treatment of diseases and inflammatory

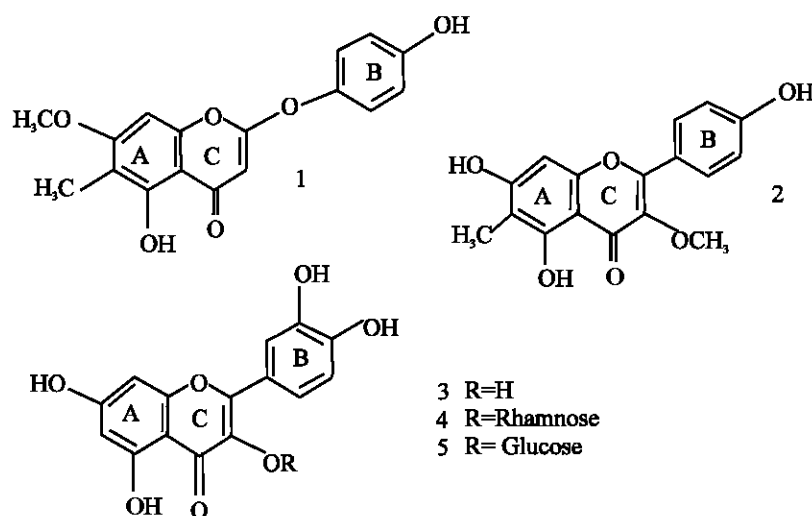


Fig. 1: Secondary metabolites from *P. reticulatum*

conditions^[4]. Fractionation of the ethyl acetate solvent fraction of the leaf extract yielded a number of secondary metabolites mainly flavonoids^[5] which include: the first ever C-methyl phenoxychromone, 2-O-(*p*-hydroxyphenyl)-5-hydroxy-6-C-methyl-7-methoxychromone (piliostigmin) (1), 6-C-methylkaempferol-3-methyl ether (2), quercetin (3), quercetrin (quercetin-3-O-rhamnose) (4), and Isoquercetrin (quercetin-3-O-glucoside)(5) (Fig. 1).

In recent years, flavonoids have gained tremendous attention as potential therapeutic agents; this is due to the fact that medicinal actions of many plants have been linked to their flavonoids constituent^[6]. For examples, promising anti-inflammatory and antibacterial activities of many have been reported^[6-8].

Quercetrin possesses antiviral activity, inhibits tumor formation, enhances insulin secretion, anti-inflammatory and antioxidant activities while quercetin has been widely reported for its preventive and curative abilities in cardiovascular and other diseases^[9]. It might be more potent in lowering cholesterol, heart disease and stroke than vitamin E^[10].

The objective of the present investigation was to evaluate the antioxidant activity of the isolated secondary metabolites from *P. reticulatum* in order to know if they could serve as natural antioxidant that could be employed in the treatment of the free radicals related diseases.

MATERIALS AND METHODS

Chemicals: All chemicals used were of analytical grade obtained from BDH Chemicals Ltd, Poole England, Fluka chemika and Sigma chemical Co. USA.

Plant material

Collection, extraction and isolation: Leaves of *P. reticulatum* were collected at Ikire, Osun State, Nigeria. Mr. G. Adesakin of the Herbarium Section, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, authenticated the plant and voucher specimen was deposited. Isolation of the chemical constituents was as previously described^[5].

Rapid evaluation of antioxidant activity by DPPH method:

The rapid evaluation of antioxidant activity of the isolated secondary metabolites from *P. reticulatum* was determined as previously described^[1] with a slight modification. Briefly, the solution of each secondary metabolite was spotted on silica gel F₂₅₄ aluminum sheets (Merck, Darmstadt, Germany) and developed in appropriate solvent system. The silica gel sheets were allowed to dry and sprayed with 0.5% solution of 1,1-diphenyl-2-picrylhydrazyl, DPPH (Fluka chemika) in

MeOH. Any spot that bleached the purple color background of DPPH reagent within 30 min was taken as positive result.

Evaluation of antioxidant activity (Quantitative determination):

The free radical scavenging activity of the isolated secondary metabolites was determined using the method of Mensor *et al.*^[11] with some modification. Briefly, isolated secondary metabolites were tested at 25.0, 12.5, 5.0, 2.5 and 1.0 $\mu\text{g mL}^{-1}$ and additional higher concentrations (70.0 and 35.0 $\mu\text{g mL}^{-1}$) were tested for compounds 1 and 2. The solution of 0.3 mM DPPH (1,1-diphenyl-2-picrylhydrazyl) in methanol was prepared and 1.0 mL of this solution was added to 2.0 mL of each sample solutions of different concentrations and allowed to react at room temperature in the dark for thirty minutes. The blank was prepared with sample solution (2.0 mL) and 1.0 mL methanol instead of the DPPH solution while the negative control was DPPH solution 1.0 mL plus methanol (2.0 mL). The decrease in absorbance was measured at 517 nm on a Visible spectrophotometer (Pharmacie Biotech, Novaspec II). These were converted to percentage antioxidant activity (AA%) using the formula:

$$\text{AA\%} = 100 - \left\{ \left[(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}) \times 100 \right] / \text{Abs}_{\text{control}} \right\}$$

Where, $\text{Abs}_{\text{sample}}$ was the absorbance of the sample, $\text{Abs}_{\text{blank}}$ was the absorbance of the blank and $\text{Abs}_{\text{control}}$ was the absorbance of the control.

The EC_{50} value, defined as the amount of the sample sufficient to elicit 50% reduction of the initial DPPH concentration, was calculated from the linear regression of plots of concentration of test compounds ($\mu\text{g/mL}$) against the mean percentage of antioxidant activity obtained from the three replicate tests. The free radical scavenging activity of ascorbic acid (Vitamin C) was also measured under the same condition to serve as antioxidant agent (positive control). This was used to compare the potency of the isolated secondary metabolites.

Data handling: The results are express as mean \pm SEM and the EC_{50} values obtained from the regression plots (SigmaPlot[®] 2001, SPSS Science) showed a good coefficient of determination ($r^2 \geq 0.934$).

RESULTS AND DISCUSSION

All the tested compounds were found to exhibit DPPH free radicals scavenging activity at varying degrees in the rapid screening. The DPPH purple colour rapidly faded when it was in contact with quercetin and its

glycosides. It took 40 and 90 seconds for 6-C-methylkaempferol-3-methyl ether (2) and 2-O-(*p*-hydroxyphenyl)-5-hydroxy-6-C-methyl-7-methoxychromone (1) to completely bleached the DPPH purple colour background, respectively. It could be safe to conclude that quercetin and its glycosides could serve as excellent alternative natural antioxidant. Compounds 1 and 2 could also have free radical scavenging activity.

From the quantitative determination, quercetin (3) demonstrated a potent antioxidant activity with good ability of scavenging DPPH free radicals. Of all the secondary metabolites and the standard investigated in this study, quercetin had the least EC₅₀ value (Table 1) and had been employed as standard agent in antioxidant studies^[1,13]. A low EC₅₀ value is an indication of strong antioxidant activity. The EC₅₀ values for the quercetin glycosides (4 and 5) were higher than that of quercetin but marginally different from that of ascorbic acid (Table 1). The higher EC₅₀ values are due to the O-glycosilation at position 3. This is in agreement with the earlier study that substitution at position 3 decreases free radical scavenging activity in flavonoids^[13].

The EC₅₀ value of piliostigmin, (1) is large and can not be determined due to the relatively high concentration required for its determination compare to the flavonoids constituent of the plant (Table 1). This suggests that the compound may be weak as antioxidant. This could be due to the presence of -O- linkage between the rings B and C. In addition, the absence of 3-OH in ring C and catechol group in ring B which enhance activity in flavonoids^[13-15] could be responsible for the large value. Piliostgmin, a rare phenoxchromone, previously obtained from *P. thonningii* did not demonstrate considerable anti-inflammatory and antibacterial activities in the earlier study^[6]. Its Minimum Inhibitory Concentration (MIC) could also not be determined, due to high concentration required for the determination.

6-C-methylkaempferol-3-methyl ether (2) had EC₅₀ value, which is lower than that of piliostgmin. This is not comparable to what obtained for the quercetin and its glycosides in this study. This could be due to methylation at position 3 and the absence of 3'-OH groups in the compound. This is in agreement with the earlier studies that the catechol group (3'-OH and 4'-OH) in ring B is the dominant factor in radical scavenging in any flavonoid that possess it^[13-15]. In both compounds 1 and 2, the role of C-methyl substitution on C-6 is not yet known. Structural requirements that has been established for good scavenging activity in flavonoids are: a catechol moiety on ring B, the 3-OH group in combination with a C2 C3 double bond and keto group in position 4^[13-15].

P. reticulatum leaves are used by diverse societies in tropical Africa for a variety of medicinal purposes. It is

Table 1: EC₅₀ values of secondary metabolites isolated from *P. reticulatum*

Compound	^a EC ₅₀ ±SEM (µg mL ⁻¹)
2-O-(<i>p</i> -hydroxyphenyl)-5-hydroxy-6-C-methyl-7-methoxychromone (piliostigmin)(1)	ND
6-C-methylkaempferol-3-methyl-ether (2)	50.76±1.71
Quercetin (3)	4.68±0.03
Quercetrin (quercetin-3-O-rhamnose)(4)	5.99±0.13
Isoquercetrin(quercetin-3-O-glucoside) (5)	6.39±0.05
Ascorbic acid (positive control)	4.97±0.06

^a Values obtained from regression plot with 95% confidence level
 ND = Not determined, EC₅₀ value of piliostgmin was not determined because it could not scavenge 50% of the DPPH free radicals under test conditions. The limiting factor was the amount of the test sample added to the DPPH reagent. SEM = Standard Error of Mean

used in the treatment of chronic ulcer and wounds, chest troubles and toothache^[4,16]. The presence of these antioxidant principles could provide rationale for the traditional uses of the plant in the treatment of the above mentioned conditions.

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