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Research Paper

Hepatoprotective and antioxidant activities of stem bark extract of *Khaya grandifoliola* (Welw) CDC and *Entada africana* Guill. et Perr.

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ABSTRACT

Khaya grandifoliola (*Meliaceae*) and *Entada africana* (*Fabaceae*) are traditionally used as source of medicines against liver related diseases. But the most efficient solvent to extract the plants bioactive compounds has not yet been found. This work aimed at evaluating and comparing the hepatoprotective and antioxidant activities of Hexane (HE), methylenechloride-methanol (MCME) and water (WE) extracts of the plants stem bark. The hepatoprotective activity was evaluated by acetaminophen-induced damage in rat liver slices in measuring lactate dehydrogenase (LDH) leakage as toxicity marker. The antioxidant activity was assessed by using 2, 4-diphenyl-1-picryl-hydrazil (DPPH), β-carotene-linoleic acid system (β-CLAMS) and microsomal lipid peroxidation (MLP) assays. The MCME extract of both plants efficiently decreased LDH leakage from liver slices. At the tested concentration of 100µg/ml, the hepatoprotective percentage of the extract of *K. grandifoliola* (87.15%) and that of silymarine (93.73%) were comparable. In the antioxidant study, the MCME and WE extracts of both plants performed well in inhibiting MLP assay but, the MCME extracts were the more antioxidant with IC_{50} values of 2.7±0.05µg/ml and 0.50±0.07µg/ml comparable with Trolox (2.27±0.52µg/ml) for *K. grandifoliola* and *E. africana*, respectively. Phytochemically, many classes of compounds including polyphenols were tested positive in the MCME extracts. From the interesting activity of these extracts, it might be concluded that methylenechloride-methanol (1:1v/v) mixture is the most efficient solvent to extract hepatoprotective and antioxidant active principles from *K. grandifoliola* and *E. africana* stem bark.

Keywords: *Khaya grandifoliola; Entada africana;* Hepatoprotective; Antioxidant.

INTRODUCTION

Pharmacological studies on *K. grandifoliola* reported the anti-inflammatory, antiulcer, hypoglyceamic, antianemia and antiplasmodial properties (Falodun, et al., 2009) of this species extracts. Phytochemical investigations conducted on the stem bark and seeds of the plant permitted to characterize limonoids (Yuan, et al., 2010) and to

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detect saponins, alkaloids, phlobatanins, carbohydrates and proteins (Ojokuku, et al., 2010).

For *E. africana,* its antileishmanial, anti-inflammatory, wound-healing and antibacterial activities have been demonstrated (Ahua, et al., 2007; Mbatchou, et al., 2011). The plant root extract is also reported to protect rat liver against CCl_4-induced damage (Sanogo, et al., 1998). Likewise, the antioxidant property of the aqueous extract of the roots, stem bark and leaf of this plant was evidenced using the singly DPPH test. Also, many phytochemical classes of compounds, including, polyphenols, terpens and alkaloids were tested present in these extracts (Tibiri, et al., 2010). Toxicity studies have revealed the safety of sub-chronic oral administration of the plant extracts in rabbit (Tibiri, et al., 2007).

Njayou et al., 2004 showed the antihepatotoxic potential activity of both plants. But, the most efficient solvent to extract hepatoprotective and antioxidant bioactive compounds from the plants is still to be found. Hence, the present investigation is to evaluate and compare the hepatoprotective and antioxidant activities of various extracts of the plants stem bark using acetaminophen-induced toxicity in rat liver slices and different *in vitro* oxidant models.

MATERIALS AND METHODS

Chemicals: All reagents used in this study were purchased from Sigma Chemicals Company (Dorset, UK) and Prolabo (Paris, France) invoice number 1029002-A.

Plant material: Stem bark of *K. grandifoliola* and *E. africana* were collected in May 2009 in Foumban (Western Cameroon). The botanical identification of the plants was done at the Cameroon National Herbarium, where voucher specimens are kept under the reference numbers 23434 YA for *K. grandifoliola* and 52661 YA for *E. africana.*

Extraction procedure: Air-dried (room temperature, 25°C) and powdered stem bark of *K. grandifoliola* (840g) and *E. Africana* (400 g) were extracted with hexane (HE) in a soxhlet apparatus in 1200 and 1000 ml of solvent respectively at 80°C for 2 hours. Each resulting extract was filtered through Whatman N°1 (England) filter paper. The filtrate was concentrated under vacuum. An oily paste obtained yielded 0.83 and 1.11% w/w respectively for *K. grandifoliola* and *E. africana*. Then, the residue of *K. grandifoliola* was divided in 2 portions of 300 and 518g and that of *E. africana* into portions of 234 and 160g. Portions of each plant residue were further extracted separately with methylenchloride-methanol (1:1v/v) mixture (MCME) and distilled water (WE) under reflux for 5 hours. The extracts dried under vacuum and oven at 40°C for aqueous extracts yielded 7.66 and 3.28; 1.68 and 5.63% w/w for MCME and WE extracts of *K. grandifoliola* and *E. africana,* respectively. Extracts kept at 4°C were dissolved in DMSO and then tested in triplicate at 1, 10, 100 and 500µg/ml. These concentrations were chosen in preliminary trials such as to be not toxic to liver slices. Hepatoprotective and antioxidant reference compounds used here were Silymarine and Trolox, respectively.

Screening of hepatoprotective activity: The hepatoprotective effect of the extracts was studied by measuring lactate dehydrogenase (LDH) leakage from rat liver slices following the procedure previously described (Njayou, et al., 2010). In a nutshell, the liver of a rat killed by decapitation was sliced into pieces of about 0.5 x 0.5 x 0.5mm, and slices were washed in a buffer and regularly aerated with a stream of oxygen for 1 hour at 37°C. Afterwards, slices distributed in capped tubes (approximatively 20-30 slices/tube) were treated with 5 µl of the plant extracts or silymarin to achieve the desired test concentrations. DMSO was used as negative control and the slices were incubated again as above for 1 more hour with regular aeration prior to intoxication

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with paracetamol (30mM) and again incubated for 2 hours. The dose of toxin chosen after preliminary trials permitted more than 50% LDH leakage from the liver slices. At the end of the 2hours, 100µl aliquot of incubation was equally removed from each tube and the content homogenized using a potter homogeniser and centrifuged (5000xg, for 5min, at 4°C). The supernatant and the previous aliquots collected were assayed for LDH activity.
The enzyme leakage percentages computed according to the following formula:

LDH leakage (%) = [(LDH Activity in S2 –LDH Activity in S0) / LDH Activity in St] x100

- Where S0: Aliquot sampled from tubes immediately after slice culture intoxication with acetaminophen, S2: Aliquot sampled from tubes at the end of 2h incubation and St: Supernatant obtained after homogenization and centrifugation of the tube content.

Screening of Antioxidant activity

Free-radical scavenging assay: The method described by Brand-William et al., 1995 was used with slight modifications. To 2ml of a methanol solution of 2, 4-diphenylpicrylhydrazyl (DPPH) 0.002% in methanol, 10µl of various concentrations of the extracts or Trolox solution (reference compound) or DMSO (blank) were added. The discoloration of the DPPH solution was monitored for 15 seconds by following the decreasing of optical density at 517nm. Then, the scavenging percentage was computed for each concentration of extracts as follows.

Scavenging activity (%)/Inhibition activity (%) = [(Absorbance of control – Absorbance of sample) / Absorbance of control] x 100 (1)

β-Carotene-Linoleic Acid Model System (β-CLAMS) assay: The test mixture was freshly prepared and used immediately (Miller, 1971). In brief, 1ml β-carotene (0.02 % w/v) dissolved in $CHCl_3$ was mixed with 20µl of linoleic acid and 200mg of tween 20. After evaporation of $CHCl_3$ using a rotary evaporator, 50ml of distilled water were added and the flask containing the mixture was shaken vigorously until the complete dissolution of the mixture. In each spectrophotometric cuve, 3ml of the mixture and 20µl of the extract, reference compound (Trolox) and blank solutions were added for test as in the above assay. The cuves were then incubated at 50°C for 3h. After this incubation, absorbance of reaction mixtures was recorded and the percentage of inhibition calculated by the formula (1).

Microsomal Lipid Peroxidation assay (MLP): Rat liver microsomes were prepared and lipid peroxidation was initiated in microsomal incubations as previously described (Njayou, et al., 2010). In short, the livers of overnight-fasted 12-week-old male Wistar rats (150-180g) were used to prepare microsomes by calcium aggregation method. Five µl of each plant extract, reference compound (Trolox) and blank solutions were tested as described above. The reaction mixture consisted of microsomes (0.4mg protein/ml), plant extract and 0.5mM ascorbate in 25mM Tris-HCl buffer, pH 7.5 containing 115mM KCl. The reaction started by the addition of 1.5µM Fe^{2+} (in the form of $(NH_4)_2Fe(SO_4)_2$) complexed with 1mM ADP. After the incubation (15 min, 37°C), the reaction stopped by the addition of thiobarbituric acid reagent. Then, samples were assayed for thiobarbituric acid-reactive substances (TBA-RS) and absorbances read at 530nm. For each concentration tested, the percentage of inhibition was computed. The amount of TBA-RS existing in the mixture before the peroxidation reaction was subtracted from the value obtained and the percentage of inhibition calculated using the equation (1).

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Phytochemical study: Standard screening tests were used to detect groups of phytochemical compounds (flavonoids, polyphenols, leucoanthocyanins, alkaloids, tannins, triterpens and sterols, anthranoids) in the extracts (Harbourne, 1984).
Statistical analysis: All data were expressed as Mean±Standard Deviation (SD). Different IC_{50}, EC_{50} values were estimated using the software graphPad prism 3.0. LDH leakage percentages were analyzed using the software MedCalc v8.0.0.1 by one way ANOVA and differences between mean values were assessed by Student Newman Keul's test, values of $P < 0.05$ were regarded as significant.

RESULTS

Hepatoprotective activity of the plant extracts: All the extracts inhibited the enzyme leakage from intoxicated liver slices in a concentration-dependent manner as shown in table 1. Although MCME and WE respective extracts of *K. grandifoliola* and *E. africana* inhibited at least 50% of the LDH leakage at 100µg/ml, the MCME extract of the former plant was as potent as silymarin at 10µg/ml in protecting rat liver slices by 70.1% from damage induced by acetaminophen.
Antioxidant activity of the plant extracts: Antioxidant activity of the plant extracts were measured using DPPH-radical scavenging, β-carotene/linoleic acid bleaching and MLP assays. All the extracts showed a concentration-dependent antioxidant activity. However, the WE extract of *K. grandifoliola,* compared to Trolox, moderately scavenged the DPPH free-radical and inhibited degradation of linoleic acid, and the plant MCME and WE extracts were both as efficient as Trolox in inhibiting MLP given their respective low IC_{50} values. Regarding *E. africana,* the MCME and WE extracts of the plant scavenged the DPPH free-radical and inhibited MLP in the same manner did the extracts of *K. grandifoliola*. However, the MCME extract inhibited MLP with a very low IC_{50} of 0.50 ± 0.07µg/ml. Results are presented in table 2.

Phytochemical classes of compounds in the extracts: Phytochemical studies of plant extracts revealed that the WE and MCME extracts of the plants studied contain the same classes of compounds. Flavonoids and polyphenols among others classes of compounds were tested positive as shown in table 3.

DISCUSSION

The rat liver slice system was validated as *in vitro* model for acetaminophen-induced toxicity studies (Elferink, et al., 2008) and successfully used to evaluate the efficacy of *Erythrina senegalensis* extracts as hepatoprotective (Njayou, et al., 2010). Acute hepatotoxicity of acetaminophen depends on the generation of a reactive metabolite that reacts with cellular and membrane components of liver cells. This reaction leads to the cellular lesion and results in the leakage of some cytosolic enzymes including LDH (Rang, et al., 1999). In our experiments, intoxication of the liver slices with acetaminophen significantly ($P<0.05$) raised the level of LDH to 56.25% in acetaminophen control compared with normal control (Table 1). The leakage of the enzyme in the incubation medium indicates that the liver slices were damaged. In this work, an extract is said to be hepatoprotective if it prevents the leakage of LDH from intoxicated rat liver slices. Pre-treatment of the liver slices with all the plant extracts at the higher tested concentration of 500µg/ml prior to acetaminophen intoxication resulted in a significant decrease of LDH leakage. However, the decreasing effect of the MCME extracts of both plants was more pronounced as compared to the activity of silymarin (Table 1). As oxidative stress plays a fundamental role in the toxicity of

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acetaminophen (Angela, et al., 2005), it is possible that the MCME extracts protected the liver slices against oxidative injury induced by acetaminophen. Thus, we evaluated *in vitro,* the antioxidative capacity of all the extracts.

Various assays are performed for determining the antioxidant activity because this activity is influenced by many factors and cannot be fully described using a single method (Wong, et al., 2006). Accordingly, we carried out three assays including the DPPH radical scavenging, β-carotene/linoleic acid bleaching (β-CLAMS) and microsomal lipid peroxidation (MLP). These systems are frequently used by researchers for the assessment of antioxidant action of medicinal plants (Chanda, and Dave, 2009). Table 2 displays the inhibitory/scavenging effects of the plants extracts on oxidative process in the studied models.

The extracts were found to have different antioxidant activity in all the assays and the poorest activity being with HE. In the DPPH test, the antioxidants reduce the DPPH radical to a yellow-colored compound, diphenylpicrylhydrazine, and the extent of the reaction will depend on the hydrogen donating ability of the antioxidants (Bondent, et al., 1997). The tested extracts demonstrated a concentration-dependent scavenging activity by quenching DPPH radicals. As compared to the activity of Trolox, the MCME extract of *E. africana* and the WE extract of *K. grandifoliola* efficiently scavenged the DPPH° radical. In this study, the results obtained concerning *E. africana* agreed with previous studies (Tibiri, et al., 2010). These authors found active the aqueous extracts of the plant in scavenging the DPPH radical after an incubation time of 15 min. After trials, the incubation period of 15 seconds was considered appropriate in our investigation. Therefore, it is possible that the plant extracts tested active here might possess high antiradicalar potential. Since the DPPH discoloration takes place by H° atoms transfer to the DPPH° radical, structural differences of chemical compounds present in each extract and bearing transferable hydrogen atome may explain the different behaviour of the extracts regarding this activity as suggested about antiradicalar efficacy of some phenolic compounds (Brand-williams, et al., 1995). So, the above-mentioned extracts may contain these types of compounds.

Lipid peroxidation is an oxidative alteration of polyunsaturated fatty acids in the cell membranes that generates a number of degradation products. During lipid peroxidation, malonaldehyde (MDA) is one of the thiobarbituric acid reactive substances (TBARS) produced and has been studied widely as an index of lipid peroxidation and as a marker of oxidative stress (Janero, 1990). Microsomal system is considered to be a better bio-membrane allowing a more thorough understanding of the interactions between plant extracts and membrane surface (Atmani, et al., 2011). Thus, in the present investigation, the membrane lipids protective effect of various extracts from *K. grandifoliola* and *E. africana* was assessed on lipid peroxidation induced by ferrous ion in rat liver microsomes and determined assaying MDA. All the extracts could prevent the formation of TBARS in a concentration-dependent manner and the MCME extracts of both plants were found more active. The lower IC_{50} values of the MCME extracts indicate a relatively high anti-lipoperoxidative activity regarding the MLP assay when compared to other plant extracts tested in the same conditions (Atmani, et al., 2011; Sathisha, et al., 2011). This suggests that the plants MCME extracts prevent the oxidation of cell membranes lipids. Lipid oxidation being a mechanism of acetaminophen-induced damage in cell membrane (Paolinelli, et al., 2006), it is possible that the inhibition of lipid peroxidation contributes to the protection afforded by the MCME extracts against toxic effects caused by acetaminophen. The iron-ascorbate system used in this study induces lipid

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peroxidation by the formation of hydroxyl radicals which attack the microsomal membrane, producing peroxyl free radicals that propagate a chain reaction (Atmani, et al., 2011). Therefore, the MLP inhibiting activity of the both MCME extracts could be attributed to their high anti-radical potential, suggesting that their constituents might be chain-terminating antioxidants.

Many papers report that antioxidant activities of plant extracts is related to the presence of phenolic compounds (Wong, et al., 2006; Moyo, et al., 2010). Preliminary phytochemical analysis conducted on the extracts revealed the presence of the same classes of compounds in the WE and MCME extracts (Table 3). This may explain why the 2 extracts relatively performed the same. But the activity of the MCME extracts was quite interesting in MLP and liver slices systems, models very closed to the *in vivo* conditions. Polyphenols such as flavonoids and tannins were among classes of compounds tested positive. Although these compounds were evidenced previously (Njayou, et al., 2004; Tibiri, et al., 2010), their absence in the HE extracts justifies its poor activity observed in the current study. Indeed, many of these phytoconstituents are known to be antioxidant, antiradicalar and hepatoprotective (Middleton, et al., 2000). The pharmacological effect of medicinal plants and extracts being attributed to their active principles, the interesting antioxidant/hepatoprotective activities exhibited by *E. africana* and *K. grandifoliola* MCME extracts in this investigation may be due to their high content in active principles.

CONCLUSION

Methylenechloride-methanol (1:1 v/v) related extracts showed the strongest hepatoprotective activity may be due to their good antioxidant demonstrated action. Therefore, in the present work, methylenechloride-methanol (1:1 v/v) mixture is likely to be the most efficient solvent among water and hexane to extract hepatoprotective and antioxidant active principles from *K. grandifoliola* and *E. africana* stem bark.

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Table-1: Effect of the plants extracts on LDH leakage from rat liver slices intoxicated with Acetaminophen.

Plant extracts Concentration (µg/ml)	LDH leakage and hepatoprotection percentages of *K. grandifoliola* extracts				LDH leakage and hepatoprotection percentages of *E. africana* extracts		
	HE	MCME	WE	Silymarin	HE	MCME	WE
1	54.67±0.99 **(2.81)**	50.25±2.35 **(10.67)**	51.49±1.79 **(8.46)**	48.61±2.48 **(13.58)**	53.51±1.25 **(4.87)**	51.25±1.58 **(8.89)**	50.09±3.68 **(10.95)**
10	43.65±0.79 **(28.96)**	23.61±1.39* **(75.01)**	26.79±1.79* **(67.71)**	23.61±1.39* **(75.01)**	47.5±3.54 **(20.11)**	41.25±1.77 **(34.47)**	42.09±2.95 **(32.54)**
100	41.43±1.43 **(34.06)**	18.33±1.67* **(87.15)**	23.61±1.39* **(75.01)**	15.47±1.19* **(93.73)**	42.47±1.81 **(31.67)**	33.75±0.00* **(51.71)**	34.11±2.28* **(50.88)**
500	35.42±2.09* **(47.87)**	13.39±0.89* **(98.51)**	20.83±4.17* **(81.41)**	12.28±1.12* **(100)**	33.04±6.74* **(53.34)**	21.25±1.77* **(80.44)**	24.88±0.41* **(72.10)**
Normal control	**12.74 ± 3.84***						
Acetaminophen control	**56.25 ± 8.83**						

- HE=Hexane Extract, MCME=MethyleneChloride-Methanol Extract, WE=Water Extract.
- Values are expressed as mean ± SD (n=4) and significantly different from acetaminophen control, *$P<0.05$.
- Numbers in parentheses indicate the percentages of hepatoprotection.

Table-2: Inhibiting/scavenging effect of the plants extracts in different oxidation models.

RC and PE	Effects of *K. grandifoliola* extracts			Effects of *E. africana* extracts		
	Oxidation models and EC_{50}/IC_{50} (µg/ml) values					
	DPPH	β-CLAMS	MLP	DPPH	β-CLAMS	MLP
Trolox	15.23± 0.57	0.66± 0.15	2.27± 0.52	15.23± 0.57	0.66± 0.15	2.27± 0.52
MCME	302.8 ± 0.08	> 500	2.7 ± 0.05	35.78 ± 0.06	152 ± 0.00	0.50 ± 0.07
WE	50.00 ± 0.08	13.86 ± 0.10	2.99 ± 0.09	81.08 ± 0.09	235.30 ± 0.02	3.53 ± 0.11
HE	NC	> 500	> 500	> 500	> 500	> 500

- Values are expressed as mean ± SD of two experiments in triplicate.
- RC and PE: Reference compound and plant extracts.
- DPPH: Free-radical scavenging assay, β-CLAMS: Inhibition of β-carotene oxidation assay, MLP: Inhibition of Microsomal Lipid Peroxidation assay. HE= Hexane Extract, MCME= MethyleneChloride-Methanol Extract, WE= Water Extract.
- NC: Value not computed because no activity was obtained with the highest dose of extract during the test.
- EC_{50}/IC_{50}: Concentration of the plant extract residue required to scavenge/inhibit 50%.

Table -3: Phytochemical class of compounds of the plant extracts.

	K. grandifoliola extracts			*E. africana* extracts		
Class of compounds	**HE**	**MCME**	**WE**	**HE**	**MCME**	**WE**
Reducing sugars	-	+	+	-	+	+
Flavonoids	-	+	+	-	+	+
Polyphenols	-	+	+	-	+	+
Tannins	-	+	+	-	+	+
Leucoanthocyanins	-	+	+	-	+	+
Triterpens	+	-	-	+	-	-
Sterols	+	-	-	+	-	-
Alkaloids	-	-	-	-	-	-
Anthranoids	-	-	-	-	-	-

- HE= Hexane Extract, MCME= MethyleneChloride-Methanol Extract, WE= Water Extract.
- (+): Positive test of the class of compounds, (-): Negative test of the class of compounds.

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