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Reversal of Coumarin-Induced Toxicity by the Extracts and Fractions of *Ageratum conyzoides*

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Abstract: This study examined the antidotal effects of the methanol extract (MeOH) and fractions of *Ageratum conyzoides* L. (Asteraceae) on coumarin-induced toxicity in rats. The effects of these extracts on coumarin acute toxicity (LD_{50}) and mortality rate were assessed. The LD_{50} of coumarin was increased by as much as 487.2 and 238.1% when co-administered with the MeOH extract (1 g/kg) and the methanol fraction, (MF) (1 g/kg) respectively. The MeOH extract and MF significantly ($p \leq 0.05$) reduced coumarin-induced mortality to 0 and 40%, respectively when compared to the 100% mortality recorded for the control group. Vitamin K and activated charcoal, which were used as standard antidotes, also produced similar reductions in percentage mortality, but MeOH extract and MF performed better. The Hexane Fraction (HF) and the ethyl acetate fraction, (EF) did not significantly ($p>0.05$) affect coumarin-induced mortality. Maximal antidotal effect was recorded when MeOH extract was given within 3 h of coumarin administration. The MeOH extract and the MF demonstrated antidotal activities against acute poisoning by coumarin in rodents. On their own, the extracts did not produce any gross pathological change in the animals, and could therefore be harnessed as useful antidote in coumarin poisoning. Local activities of the phytoconstituents of *A. conyzoides* on the GIT which limits absorption and haemorrhagic damages are believed to be responsible for the reversal of the coumarin toxicity.

Key words: *Ageratum conyzoides*, antidote, coumarin, coumarin- toxicity, poisoning

INTRODUCTION

Coumarin is a toxic chemical compound (benzopyrone) found in many plants, notably in high concentration in the tonka bean, woodruff, mullein, and sweet clover (Majerus and Tollefsen, 2006). Toxicity from coumarins was first observed in animals. Livestock that were fed moldy spoiled sweet clover hay died from a previously unknown hemorrhagic disorder. Bishydroxycoumarin, the active ingredient responsible for this hemorrhagic disorder, was discovered by Campbell and Link (1941). Bishydroxycoumarin is formed when fungi in moldy sweet clover oxidize coumarin to 4-hydroxycoumarin, an anticoagulant. Bishydroxycoumarin was synthesized and used clinically one year later as an oral anticoagulant under the American trade name dicumarol (Campbell and Link, 1941). Although only somewhat dangerous to humans, coumarin is a potent rodenticide. Rats and other rodents largely metabolize it to 3, 4-coumarin epoxide, a toxic compound that can cause internal hemorrhage and death (Cain *et al*., 1997). Humans largely metabolize it to 7-hydroxycoumarin, a compound of lower toxicity (Kaminsky and Zhang, 1997).

Ageratum conyzoides L. (Asteraceae), commonly called tropical whiteweed, Billy goat weed, or floss flower and also synonymously known as *A. latifolium* L., *A. cordifolium* L., *A. album* L.and *A. odoratum* L. is a common tropical annual herbaceous weed which grows up to a height of 2.5 feet with little white flowers and opposite leaves (Dalziel, 1958). The stem is often reddish and has long white hairs. The weak aromatic unpleasant smelling leaves are also covered with fine hair. *A. conyzoides* is thought to be native to South America, but has spread to the tropical and subtropical regions.

A. conyzoides is used in folk medicine for various ailments in Africa (Almagboul *et al*., 1985), Asia, and South America (Ekundayo *et al*., 1988). The common medicinal uses of *A. conyzoides* in West Africa is for wound healing, especially burns (Watt and Breyer-Brandwijk, 1962). Ageratum is also used as an antiepileptic, antihelminthic, and as an insect repellent (Adodo, 2004). *A. conyzoides* has also been reported in the treatment of Human Immunodeficiency Virus (HIV) and acquired immunodeficiency syndrome among the Igede community of Benue State, Nigeria (Igoli *et al*., 2005). Some pharmacological properties have also been associate with *A. conyzoides* such as haemostatic (Akah, 1988), muscle relaxant (Achola *et al*., 1994), antinflammatory (Maglhaes *et al*., 1997), antimicrobial (Almagboul *et al*., 1985; Akinyemi *et al*., 2005;

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Amadi *et al*., 2007), glucose lowering effect (Nyunai *et al*., 2006), and gastroprotective activity (Shirwaikar *et al*., 2003).

A variety of phytochemical compounds have been identified in the tropical whiteweed. These include alkaloids, coumarins, essential oils, flavonoids and tannins (Horiet *et al*., 1993; Adeleke *et al*., 2002). The essential oil of *A. conyzoides* is composed of phenols, phenolic esters, coumarin, β-cariophylene and ageratochrome (Oliver-Bever, 1986; Vera, 1993). The African species of *A. conyzoides* contain eugenol as the major essential oil (Ekundayo *et al*., 1988).

In Igboukwu Community of South Eastern Nigeria, the plant is popularly used to arrest bleeding from fresh wounds and to stop epitasis. Also victims of various poisons are made to chew copious quantity of the fresh leaves of *A. conyzoides*, a practice that has been reputed to result in high degree of antidotal efficacy. This study is aimed at evaluating the antidotal effects of the leaf extracts of *A. conyzoides* on coumarin-induced toxicity in rodents.

MATERIALS AND METHODS

Animals: Adult albino rats (100-200 g) and adult albino mice (21-25 g) of both sexes obtained from the Laboratory Animal Facility Centre of the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka were used in the study. The animals were housed under standard conditions (25±2ºC and 12 h light/dark cycle). The rats and mice were maintained on standard pellets (Livestock feed®), and allowed unrestricted access to drinking water. The use and care of laboratory animals were conducted in accordance with the internationally accepted best practices as contained in the European Community guidelines (EEC Directive of 1986; 86/609/EEC). These studies were carried out in 2007 in the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka (UNN), Nigeria.

Preparation of plant material: The fresh leaves of *A. conyzoides* were collected in Igboukwu, South Eastern Nigeria in the month of July, 2005 and were authenticated by Mr. J.M.C. Ekekwe, of the Department of Botany, University of Nigeria, Nsukka, where a voucher specimen has been deposited. The leaves were air-dried under shade and then pulverized with a laboratory mill. The leaf powder (3 kg) was divided into two portions. The first portion (1 kg) was extracted with methanol in a soxhlet extractor to obtain the methanol extract (MeOH) and the second portion (2 kg) was successively extracted with n-hexane (HF), ethylacetate (EF) and methanol (MF) using soxhlet extractor to obtain three solvent fractions. Preliminary phytochemical studies were carried out on the leaf powder and on the extracts using standard procedures (Harbourne, 1984).

Acute toxicity (LD_{50}) test of the extracts and fractions: The acute toxicity profile of the extracts and fractions of *A. conyzoides* was estimated in mice using method described by Lorke (1983). Briefly, the tests involved two phases. The first phase is the determination of the toxic range. The mice were placed in three groups of three mice each. The groups were administered the extracts intraperitoneally (ip) at the doses of 10, 100, and 1000 mg/kg respectively. The extracts were solubilized in a solution of 3% Tween 80 in water. The treated mice were observed for 24 h for the number of deaths.

The death pattern in the first phase determined the doses used for the second phase according to Lorke (1983) estimation. In this phase, four groups of one mouse each were used for each extract tested. Each group received suitable doses of the extract i.p. as predetermined in the first phase and the animals observed for lethality or signs of acute intoxication for 24 h. The LD_{50} was calculated as the geometric mean of the highest non-lethal dose and the least toxic dose (Lorke, 1983).

Antidotal tests:

Lethality of coumarin: The acute toxicity of coumarin was assessed by determining its LD_{50} as described in the previous section (Lorke, 1983).

Effect of the extracts on the lethality (LD_{50}) of coumarin in mice: The LD_{50} of coumarin was re-evaluated after the animals were pretreated orally with graded doses (100, 250, 500, 1000, and 5000 mg/kg) of the extracts.

Effect of the extracts on the lethality of coumarin in rats: The effect of *A. conyzoides* extracts on coumarin toxicity was also studied in eight groups of rats (n/gp = 6). The animals were fasted for 12 h prior to the experiment, but were allowed access to drinking water. The minimum lethal dose of coumarin (600 mg/kg) as previously determined was administered orally to the animals in the groups. Five groups were immediately given graded doses of the extracts (100-5000 mg/kg) orally while three groups were treated orally (in two divided doses) with activated charcoal (6000 mg/kg), Vitamin K_1 (5 mg/kg), vehicle (3% Tween 80, 1 ml/rat) respectively. The animals were then observed for 48 h and the number of deaths recorded as percentage mortality.

Time-dependent effect of the methanol extract on coumarin toxicity: The MeOH extract, which was found most active in the previous procedures, was used in this study. Six groups (n/gp = 6) of albino rats were employed. The rats were administered orally once with 600 mg/kg of coumarin followed by 1000 mg/kg of the MeOH extract at 0, 1, 3, 6, 12, and 24 h to the different groups respectively. The animals were observed for 48 h for deaths and the percentage mortality recorded.

Table 1: Phytochemical constituents and extractive yield of *A. conyzoides*

Extract	Yield (g)[#]	Phytoconstituents
MeOH	123 (12.3)	Alkaloids, saponins, tannins, glycosides, flavonoids, resins, protein, carbohydrate, reducing sugars, steroids, and terpenoids
HF	70 (3.5)	Alkaloids, flavonoids, resins, carbohydrate
EF	86 (4.3)	Alkaloids, flavonoids, resins, carbohydrate
MF	134 (6.7)	Alkaloids, saponins, tannins, glycosides, flavonoids, resins, protein, carbohydrate, reducing sugars

[#]: Percentage yield in parenthesis

Table 2: Effect of the extract and fractions of *A. conyzoides* on the LD_{50} of coumarin

Dose (mg/kg) of extract	LD50 of coumarin (mg/kg)			
	MeOH	HF	EF	MF
100	367.42 (26.8)	282.84 (-2.4)	289.83 (0.0)	288.53 (-0.5)
250	471.12 (62.6)	289.83 (0.0)	288.53 (-0.5)	400.00 (38.0)
500	1264.91 (336.4)	113.14 (-61.0)	227.60 (-21.5)	565.69 (95.2)
1000	1319.09 (355.1)	80.0 (-72.4)	200.0 (-31.0)	979.80 (238.1)

Percentage increases in the LD_{50} of coumarin (289.83 mg/kg) are shown in parenthesis

Statistical analysis: Results obtained were analyzed using one way analysis of variance (ANOVA) followed by Fischer LSD *post hoc* test and were expressed as mean ± standard error of the mean (SEM). Differences between paired mean observations were regarded as significant at $p \leq 0.05$. The SPSS statistical software (version 11.0) was used.

RESULTS

Phytochemical test: Phytochemical studies showed that the leaves of *A. conyzoides* contain alkaloids, tannins, flavonoids, saponins, resins, carbohydrate, protein, fats and oil. The occurrence of these metabolites in the different extracts and fractions are shown in Table 1.

Acute toxicity (LD_{50}) of the extracts of *A. conyzoides*: The intraperitoneal (ip) LD_{50} were estimated as 1265, 2150, and 3 810 mg/kg for HF, EF, and MF, respectively. The MeOH extract administered up to a dose of 5 g/kg did not cause any mortality in the mice.

Lethality (LD_{50}) of coumarin: The intraperitoneal (ip) LD_{50} of coumarin was estimated to be 289.83 mg/kg. The animals that died within 24 h of the experiment showed signs of coumarin anticoagulation toxicity. These signs include fatigue, anorexia, haematuria, bruises, melena, reduced activity and eventual death.

Effect of the extract and fractions on the LD_{50} of coumarin: There was a dose-related increase in the LD_{50} of coumarin when co-administered with the MeOH extract and MF (Table 2). The LD_{50} of coumarin was increased for as much as 487.2 and 238.1% when the animals were co-administered with the methanol extract (5 g/kg) and the methanol fraction (1 g/kg) respectively. Generally, the HF caused a dose-dependent decrease in the LD_{50} of coumarin at doses above 500 mg/kg. The EF did not affect the LD_{50} of coumarin at doses below 1000 mg/kg, but caused significant decreases at doses above 1000 mg/kg (Table 2).

Table 3: Effect of the extract and fractions of *A. conyzoides* on coumarin-induced mortality

Treatment	Dose (mg/kg)	Mortality (%)
MeOH	100	60
	250	60
	500	20
	1000	0
	5000	0
HF	100	100
	250	80
	500	100
	1000	NT
	5000	NT
EF	100	100
	250	100
	500	80
	1000	100
	5000	NT
MF	100	80
	250	60
	500	40
	1000	40
	5000	NT
Activated charcoal	6000	60
Vitamin K	5 mg/kg	40
Tween 80	1 ml	100

NT represents the doses that were not tested considering the LD_{50} of the respective extract

Effect of the extract and fractions on coumarin-induced mortality: The study showed that only MeOH extract and MF exhibited significant ($p \leq 0.05$) antidotal effect as indicated by the decrease in percentage mortality recorded when the rats were pretreated with these extracts (Table 3). Vitamin K and activated charcoal, which were used as standard antidotes in the study, produced similar decreases in percentage mortality, but MeOH extract and MF performed better. The HF and EF did not affect coumarin-induced mortality significantly when compared to the negative control. The MeOH extract performed better than all other extracts tested (Table 3).

Table 4: Time-dependent effects MeOH extract of A. conyzoides on coumarin toxicity

		Mortality (%)					
Treatment	Dose (mg/kg)	0 h	1 h	3 h	6 h	12 h	24 h
MeOH	1000	0	0	0	60	100	50
Activated charcoal	6000	40	40	60	80	100	100
Vitamin K	5	20	20	40	20	40	40

Time-dependent effect of MeOH extract on coumarin toxicity: The maximal antidotal effect was recorded when the MeOH extract was given within 3 h of coumarin administration, thereafter there was a gradual loss of the protection as shown by increased mortality rate (Table 4).

DISCUSSION

In this study, the methanol extract and the methanol fractions of *Ageratum conyzoides* exhibited antidotal properties against coumarin-induced toxicity since they caused significant ($p \leq 0.05$) increase in the LD_{50} of coumarin when co-administered. Although both share some similarities in phytochemical constituents, the methanol extract showed higher antidotal activities than the methanol fraction. Higher levels of active constituents, which are responsible for the antidotal properties, could explain this observation. It could also be explained by the better tolerability of the MeOH extract by the animals as compared to MF. In our toxicity experiment, the methanol extract could be considered safe, since the mice tolerated dose up to 5 g/kg body weight without mortality or signs of acute intoxication (Lorke, 1983).

It is interesting to observe that the antidotal effect of the methanol extract of *A. conyzoides* was quite comparable to that shown by activated charcoal, a locally acting adsorbent-antidote commonly used clinically in acute substance-intoxication. We also performed a time-of-intervention study to assess the antidotal effect over different time of administration. In the study, amelioration of coumarin toxicity by the extract of *A. conyzoides* was only remarkable when it was given not later than 3 h of coumarin administration. Early administration before the third hour of coumarin administration resulted in zero mortality rates in the methanol extract treated group. The mortality rate increased with increase in the elapsed time before intervention. This result suggests that the extract may have a local antidotal action, acting early to prevent absorption or to protect gastric mucosa against hemorrhagic lesions. Coumarin is known to be absorbed slowly, which could take up to the 3 h (Hoult and Paya, 1996). Administration of the extract at 6 h after coumarin did not produce significant antidotal protection. This also supports our earlier assertion on a possible local action even though more studies is needed to rule out possible systemic effects. Unlike the methanol extract of *A. conyzoides*, the antidotal effectiveness of Vitamin K was more pronounced when given 6 h after coumarin administration. Coumarins inhibit hepatic synthesis of the Vitamin K-dependent coagulation factors II, VII, IX, and X and the anticoagulant proteins C and S (Colman *et al*., 2005). Vitamin K is a cofactor in the synthesis of these clotting factors. The Vitamin K-dependent step involves carboxylation of glutamic acid residues and requires regeneration of Vitamin K to its reduced form. Coumarins and related compounds inhibit Vitamin K_1-2, 3 epoxide reductase, preventing Vitamin K from being reduced to its active form (Colman *et al*., 2005). The result of the study with Vitamin K in the reversal of coumarin toxicity is therefore systemic.

The administration of the methanol extract and the methanol fraction of *A. conyzoides* increased the LD_{50} of coumarin significantly ($p \leq 0.05$), this demonstrates the potent antidotal effect of the extracts in coumarin poisoning. We have estimated the oral LD_{50} of coumarin to be 289.83 mg/kg, quite comparable to an earlier estimate of 290 mg/kg (Hazleton *et al*., 1956).Resin-drug complexes have been used extensively in drug delivery systems to delay absorption and achieve sustained release characteristics (Woodworth *et al*., 1992; Wen *et al*., 1997; Umamaheshwari *et al*., 2003). It is possible that the abundance of resinous substances in the methanol extract and fraction could be responsible for the antidotal activity since resins are capable of complex formation with wide range of molecules which could delay absorption.

The presence of resins may not fully explain the reversal of coumarin toxicity by the extracts since the hexane and ethyl acetate fractions which also contain resins did not show remarkable antidotal effects. Coumarin is a plant glycoside and tannins are known to precipitate glycosidal drugs and poisons (Hoult and Paya, 1996). It is therefore possible that the presence of tannins in the methanol extract and fraction could also partly or wholly explain the observed antidotal activity. The major site of anticoagulation in coumarin is the gastrointestinal tract leading to haemorrhagic lesions. Antioxidants have been shown to be gastroprotective and to alleviate such haemorrhagic damages (Ligumsky *et al*., 1995; Mahmood *et al*., 2005). Flavonoidal compounds isolated from *A. conyzoides* and the aqueous extract from the plant have been shown to possess significant antioxidant properties in a previous study (Gonzalez *et al*., 1991). This effect could also have contributed to the reduced mortality and increased LD_{50} caused by *A. conyzoides* in coumarin-treated rats.

In an earlier study (Akah, 1988), the aqueous extract of *A. conyzoides* was reported to have haemostatic activity by two mechanisms. Firstly, the precipitation of blood constituents as a result of its high tannin content, which results in the formation of an artificial clot to arrests bleeding. Secondly, the vasoconstrictive action of the extracts of *A. conyzoides* was also identified. These same mechanisms may also occur locally in the gastrointestinal tract to protect the animals. Our preliminary study had identified the gastrointestinal tract as the major site of coumarin haemorrhagic toxicity.

The methanol extract and the methanol fraction of the leaves of *A. conyzoides* demonstrated antidotal activities against acute poisoning by coumarin in rodents and could therefore be harnessed as a useful antidote in coumarin poisoning. A combination of several mechanisms which are believed to be localized in the GIT may have contributed in the reversal of the coumarin toxicity. This study could offer explanation to the successes claimed by herbalists in their use of *Ageratum conyzoides* extracts as a general antidote in the treatment of different kinds of poisoning.

CONCLUSION

The results of this study revealed that the extracts of *Ageratum conyzoides* demonstrated potent antidotal activity against acute coumarin poisoning. Local activities of the secondary metabolites present in the extract, which limits absorption, and hemorrhagic damage of the gastrointestinal tract may be responsible for the reversal of coumarin toxicity.

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