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Toxicological and Pharmacological Studies of New Coumarin and Furocoumarin Derivatives in Albino rats

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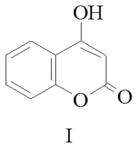
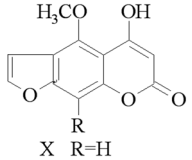
Abstract – Synthetic coumarin and furocoumarin derivatives were evaluated for anticoagulant activity and the effect on liver and kidney function. It was found that all of the compounds under investigation proved to be neither toxic nor lethal up to 500 mg/100 g body weight as a single dose for 24 hrs. All tested compounds showed a significant increase in prothrombin time (PT) in the acute model but failed to show a significant action in the chronic model. Furthermore, all tested compounds revealed a significant increase in activated partial thromboplastin time (APTT) as compared to control value in both acute and chronic model. Also, all tested compounds did not cause any significant changes on liver and kidney functions in rats.
Keywords – Coumarin, Furocoumarin, PT, APTT, AST, ALT, Urea and Creatinine

Introduction

A variety of biologically active compounds are derived from coumarin and furocoumarins (Sethna and Shah, 1945; Feuer, 1973; Cremyln *et al.*, 1978). Furocoumarins are found especially in the umbelliferae, rutaceae, leguminosa families and also in others families of lesser importance (Mustafa, 1967). 4-hydroxycoumarin (I) comprises the structural nucleus of many natural products, drugs and pesticides (Obaseki and Porter, 1982). It is the key intermediate for various broadly used oral anticoagulants and rodenticides (Hermodson *et al.*, 1971). On the other hand, it is also widely realized that the biological activity of many drugs is associated with or enhanced by the presence of a furan nucleus in their molecules (Mustafa, 1967; Murray *et al.*, 1982). In view of the a forementioned information, it seemed of interest to design and synthesise new furocoumarin derivatives which incorporate both the furan nucleus and the 4-hydroxy coumarin residue. This designing approach has also been stimulated by the fact that many furo coumarins are known to attract much interest as photochemotherapeutic agents in treatment of some skin disease such as psoriasis and vitiligo (Fahmy and Abu Shady, 1948; Luftl *et al.*, 1997; Gasparro *et al.*, 1998). They act as hepatoprotective (He *et al.*, 1998), anti-inflammatory; anti-allergic (Yamahara *et al.*, 1985; Kimura and Okuda, 1997), antimicrobial and anti-HIV (Manderfeld *et al.*, 1997), antimitotic and anticancer (Okuyama *et al.*, 1990; Nishino *et al.*, 1990; Wu, 1992), antianginal and antiarrhythmic (Occhiuto and Circosta, 1997), antidepressant (Bergendorff *et al.*, 1997) and also as effective photoreactive croos-linkage reagents for nucleic acids (Perkins *et al.*, 1999). The sequence of reaction followed for the preparation of the designed compounds was performed and provided by the organic chemistry lab. N.R.C. Cairo Egypt. Which are prepared by the Michael condensation reaction of 4-hydroxy coumarin (I) with appropriate α, β-unsaturated carbonyl compound (Yakout *et al.*,1998 and Yakout *et al.*,1999). A literature survey however, revealed that the reaction of both 4-hydroxycoumarin (I) and compounds incorporating a similar structural unit, e.g. 4-hydroxy-bergapten (x) and 4-hydroxyisopimpinellin (XI) with a,b-unsaturated nitriles, has not yet been investigated. Therefore, it appeared of interest to study the reaction of I, X and XI with the nitrile synthons $II_{(1-10)}$ in the search for new N-and O-heterocycles of promising biological activities.



I



X R=H
X1 R= OCH_3

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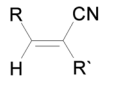
Reaction of 4-hydroxy coumarin (I) with α, β-unsaturated nitriles $II_{(1-10)}$ proceeds in absolute ethanol to give 1 : 1 adducts for which structure $III_{(1-10)}$ were assigned.

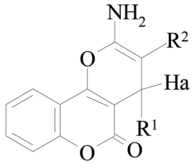
Under similar conditions the reaction of X and XI with α, β-unsaturated nitriles (II_{1-10}) afforded the respective pyranofurocoumarins (XII_{1-20}).

Also, reaction of X and XI with aldehydes $XV_{(1-7)}$ in prescence of thiophenol to give $XVIII_{(1-14)}$.

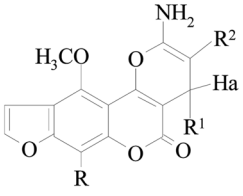
This work aimed to evaluate the effect of some of the tested compounds on toxicological and pharmacological activity.



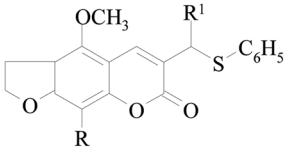
II



$III_{(1-10)}$



$XII_{(1-20)}$



$XVIII_{(1-14)}$

Table (1):

	R	R'	R''
A	H	C_6H_5	CN
B	OCH_3	C_6H_5	CN
C	H	C_6H_5	$CO_2C_2H_5$
D	OCH_3	C_6H_5	$CO_2C_2H_5$
E	H	C_6H_4-OCH_3-P	CN
F	OCH_3	C_6H_4-OCH_3-P	CN
G	H	C_6H_4-NO_2-m	CN
H	OCH_3	C_6H_4-NO_2-m	CN
I	H	C_6H_4-NO_2-p	CN
J	OCH_3	C_6H_4-NO_2-p	CN
K	H	C_6H_4-NO_2-p	$CO_2C_2H_5$
L	OCH_3	C_6H_4-NO_2-p	$CO_2C_2H_5$
M	H	C_6H_4-OH-p	CN
N	OCH_3	C_6H_4-OH-p	CN
O	H	C_6H_4-OH-o	CN
P	OCH_3	C_6H_4-OH-o	CN
Q	H	C_4H_3O	CN
R	OCH_3	C_4H_3O	CN
S	H	C_4H_3S	CN
T	OCH_3	C_4H_3S	CN

Materials and Methods

Animals – Mice of both sexes aged (3 - 6 months), twelve groups (4 mice each) and rats of both sexes 150 ± 10 g of body weight were obtained from the animal houses colony, National Research Centre. Animals were housed in plastic ages with stainless steel cover, provided with standard laboratory diet and water freely and housed under standard housing conditions. All animal procedures were performed in accordance with the recommendations for the proper care and use of laboratory animals (NIH Publ. No. 85-23, rev. 1985).

A. Acute toxic effect – The aim of this study is to determine the possible acute toxic effect of the newly synthesised compounds were orally (P.O) administered to mice as a single dose for 24 hours, LD_{50} of the different tested compounds were determined according to the method described by Karber (1931).

B. Pharmacological screening – To investigate the possible anticoagulant effect of the tested compounds, rats were divided into thirteen groups "6 rats each". Rats in group (1) were kept as control and received 10% propylene glycol in distilled water only, whereas those of groups from (2-12) were given the tested compounds XII_1, XII_2, III, XII_3, XII_{10}, XII_{14}, III_5, $III_{6,}III_{10}$, $XVIII_1$ and $XVIII_8$, rats in the last group (13) were given warfarin (5 mg/100 g.b.wt.), Heneghan *et al.,* 2006 as standard positive control. Tested doses of the tested compounds were $1/10^{th}$ of the maximal non toxic dose obtained from the acute toxicity study. Tested doses were given orally to rats for five successive days. Blood samples for hematological investigation were taken on 33.3% solution of sodium citrate. The first two samples were taken on the 2^{nd} and 5^{th} days of administration of the tested compounds, while the third one was taken three days after the last day of treatment (8^{th} day).

C. Effect of prolonged administration – Some of the pharmacologically tested compounds "XII_1, XII_{10}, XII_{14}, III_5, XII_3, XII_3" were tested for their effect on prolonged administration of selected dose ($1/20^{th}$ of the maximal non toxic dose) given orally for 21 days in rats to evaluate their possible toxic effect. Blood sample were taken on the 7^{th}, 14^{th} and 21^{st} days of administration. The blood samples were taken. Serum was separated and the following parameters were tested.

Parameters – Prothrombin time (PT) by (Caen *et al.*, 1975). Bio Merieux (Lyon, France) kits were used for the determination of prothrombin time.

Activated partial thromboplastin time (APTT) (Dacie and Lucie, 1991).

Table 1. Means ± SD of (PT/sec) and (APTT/sec) of rats treated with tested compounds (50 mg/100 g body weight) compared to that of control

Compound	Time of sampling 'days' after beginning of administration					
	2nd		5th		8th #	
	PT/sec	APTT/sec	PT/sec	APTT/sec	PT/sec	APTT/sec
Control	16.7 ± 0.62	24.6 ± 3.5	16.1 ± 1.33	24.3 ± 3.5	17.3 ± 1.4	26.2 ± 5.4
XII_1	16.2 ± 1.7	21.8 ± 1.7	60.0 ± 0.00**	61.0 ± 6.8**	66.0 ± 0.00**	66.0 ± 0.0**
XII_2	18.6 ± 1.41	24.7 ± 4.1	22.2 ± 6.6*	30.8 ± 12.4	21.8 ± 4.8*	27.4 ± 2.9
III_1	19.3 ± 2.5*	24.3 ± 4.9	17.8 ± 0.8*	23 ± 3.1	18.1 ± 0.8	24.2 ± 4.2
XII_3	35.5 ± 15.9**	45.8 ± 16.7*	35.6 ± 7.7**	41.5 ± 16.4**	23.2 ± 4.7	24.5 ± 3.4
XII_{10}	20.9 ± 2.5*	24.2 ± 2.7	26.7 ± 6.1*	36.3 ± 5.3**	26.4 ± 5.2*	35.2 ± 10.3*
XII_{14}	58.8 ± 9.8*	62.6 ± 7.6**	58.6 ± 8.0**	62.3 ± 6.3**	66.0 ± 0.0**	66.0 ± 0.0**
III_5	20.8 ± 1.7*	31.6 ± 7.8*	21.4 ± 2.8*	29.8 ± 7.8*	20.8 ± 1.3	27.6 ± 5.5
III_6	20.1 ± 2.5*	26.7 ± 4.4	20.6 ± 1.9*	20.7 ± 1.1	17.5 ± 0.8	23.0 ± 1.0
III_{10}	18.4 ± 0.4**	26.4 ± 5.7	18.5 ± 0.4*	26.2 ± 3.2	22.7 ± 4.6*	29.0 ± 8.7
$XVIII_1$	21.4 ± 1.1**	26.4 ± 2.1	21.7 ± 2.3*	26.0 ± 3.4	22.6 ± 3.8	30.7 ± 7.6
$XVIII_8$	21.8 ± 2.0*	25.0 ± 2.7	22.4 ± 2.1**	25.0 ± 3.9	21.5 ± 1.9*	25.4 ± 7.8
Warfarin 5 mg/100 g.b.wt	60.2 ± 2.6**	70.2 ± 16.9**	All died	All died	All died	All died

*Significant difference between compound and control ($P < 0.05$)
**Significant difference between compound and control ($P < 0.01$)
3 days after stopping administration.

The activities of serum aspartate aminotransferase (AST) and serum alanine aminotransferase (ALT) were determined using autoanalyzer Express Plus-Ciba corning according to the method of (Horder and Rej 1983).

Determination of serum blood urea nitrogen and creatinine both were determined using autoanalyzer Express Plus-Ciba corning following the method of Lespinas *et al*., 1989 for BUN and the method of Spencer (1986) for creatinine.

Results

Effects of acute toxicity – It was found that all of the compounds under investigation proved to be neither toxic nor lethal up to 500 mg/100g b.wt. as a single oral dose for 24 hours.

Pharmacological screening:

1. Prothrombin time (PT) – The mean value of PT are listed in Table 1. In the fifth day of administration, PT significantly increased in treated-rats with all tested compounds as compared to that of control value.

2. Activated partial thromboplastin time (APTT) – The results of APTT obtained after administration of the tested compounds are tabulated in Table 1. They show that in rats treated with compounds XII_1, XII_3, XII_{10}, XII_{14} and III_5, the APTT increased significantly (5th and 8th), (2nd and 5th), (5th and 8th), (2nd, 5th and 8th) and (2nd and 5th) days, respectively.

Effect of prolonged administration:

1. Prothrombin time (PT) – The mean values for PT are listed in Table 2. They show that treated rats with compounds XII_1, XII_{10} and III_5 insignificantly increased PT as compared with that of the control group.

2. Activated partial thromboplastin Time (APTT) – The results of APTT obtained during administration of tested compounds are tabulated in Table 2. The results revealed that the mean values of APTT increased significantly with compounds XII_1, XII_{10}, XII_{14}, and III_5 treated rats after (1st) (1st and 2nd), (1st , 2nd and 3rd) and (1st and 2nd) weeks from the beginning of administration, respectively. In XII_3 treated-rats the APTT was significantly higher after the first week of administration at the two doses illustrated in tables. Then promptly, all rats died after 2 and 3 weeks.

3. Serum Transaminases (AST) and (ALT) – The mean values of liver enzymes AST and ALT are tabulated in Tables 3. All the tested compounds did not produce any significant changes in AST or ALT all over the period of the experiment. However in case of treated-rats with compound XII_3 (in a dose 12.5 mg/100 g b.wt), the AST increased significantly after the first week from the beginning of administration.

4. Serum blood urea Nitrogen BUN and creatinine – The mean values of BUN and creatinine are tabulated in Tables 4 they did not show any significant changes all-over the period of the experiment in all the tested compounds when compared to those of control.

Table 2. Means ± SD of (PT/sec) and (APTT/sec) of rats treated with tested compounds (25 mg/100 g body weight) compared to that of control

Compound	Time of sampling 'weeks' after beginning of administration					
	1st		2nd		3rd	
	PT/sec	APTT/sec	PT/sec	APTT/sec	PT/sec	APTT/sec
Control	20.5 ± 1.2	22.7 ± 2.3	17.8 ± 0.75	22.8 ± 2.4	18.0 ± 1.4	26.2 ± 5.8
XII_1	19.2 ± 2.3	52.7 ± 11.6*	23.2 ± 13.2	33.8 ± 16.4	29.3 ± 13.2	27.2 ± 3.9
XII_{10}	17.8 ± 0.8	34.6 ± 7.9*	18.2 ± 0.6	29.2 ± 1.6	20.7 ± 1.4	29.5 ± 11.2
XII_{14}	22.3 ± 6.7	36.8 ± 13.5*	20.5 ± 8.6	30.2 ± 5.2*	29.4 ± 21.9	42.2 ± 14.5*
III_5	19.6 ± 1.1	31 ± 4.2*	21.3 ± 2.1	34.2 ± 4.8*	19.2 ± 0.9	25.2 ± 3.7
XII_3	66.0 ± 0.0**	66.0 ± 0.0**	All died	All died	All died	All died
XII_3•	53.0 ± 1.52**	63.0 ± 7.3**	All died	All died	All died	All died

*Significant difference between compound and control ($P < 0.05$)
**Significant difference between compound and control ($P < 0.01$)
•The dose given is 12.5 mg/100 g body weight

Table 3. Means ± SD of serum AST and ALT (IU/L) of rats treated with tested compounds (25 mg/100 g body weight) compared to that of control

Compound	Time of sampling 'weeks' after beginning of administration					
	1st		2nd		3rd	
	AST	ALT	AST	ALT	AST	ALT
Control	167.5 ± 19.6	43.8 ± 10.6	174.3 ± 54.9	46 ± 3.2	158 ± 21.9	31.6 ± 5.7
XII_1	146.2 ± 41.2	36.8 ± 3.1	168.3 ± 16.9	45.3 ± 11.1	157.5 ± 33.5	40.7 ± 13.9
XII_{10}	136.5 ± 21.2	40.2 ± 6.5	138.5 ± 35.1	39.2 ± 6.2	152 ± 39.5	34 ± 4.7
XII_{14}	172.4 ± 35.4	44.6 ± 8.5	155.8 ± 28.8	43.2 ± 5.3	163.2 ± 24.5	31.0 ± 2.9
III_5	145.2 ± 8.2	39.6 ± 7.1	181.6 ± 35.1	43.6 ± 9.3	150.2 ± 25.8	41.2 ± 6.5
XII_3	157 ± 0.0	50 ± 0.0	All died	All died	All died	All died
XII_3•	195.0 ± 24.1**	53 ± 15.2	All died	All died	All died	All died

*Significant difference between compound and control ($P < 0.05$)
**Significant difference between compound and control ($P < 0.01$)
•The dose given is 12.5 mg/100 g body weight

Table 4. Means ± SD of serum urea nitrogen (mg/dl) and creatinine (mg/dl) of rats treated with tested compounds (25 mg/100 g body weight) compared to that of control

Compound	Time of sampling 'weeks' after beginning of administration					
	1st		2nd		3rd	
	Urea	Creatinine	Urea	Creatinine	Urea	Creatinine
Control	25.5 ± 8.8	0.5 ± 0.08	33.0 ± 4.1	0.5 ± 0.0	32.5 ± 6.4	0.4 ± 0.0
XII_1	23.6 ± 3.1	0.44 ± 0.05	33.3 ± 3.6	0.5 ± 0.15	31.4 ± 4.4	0.44 ± 0.08
XII_{10}	27.3 ± 5.1	0.43 ± 0.05	27.6 ± 4.2	0.43 ± 0.05	30.2 ± 4.4	0.4 ± 0.0
XII_{14}	26.0 ± 3.8	0.56 ± 0.05	23.2 ± 2.7	0.42 ± 0.04	31.5 ± 0.4	0.47 ± 0.09
III_5	22 ± 1.6	0.42 ± 0.04	23.3 ± 1.5	0.43 ± 0.05	28.2 ± 4.2	0.42 ± 0.0
XII_3	22 ± 0.0	0.5 ± 0.0	All died	All died	All died	All died
XII_3•	27.5 ± 2.1	0.4 ± 0.0	All died	All died	All died	All died

*Significant difference between compound and control ($P < 0.05$)
**Significant difference between compound and control ($P < 0.01$)
•The dose given is 12.5 mg/100 g body weight

Discussion

The results revealed that compounds XII_1, XII_3, XII_{10}, XII_{14}, and III_5 induce a prolongation in PT and APTT while XII_2, III_1, III_6, XII_{10}, $XVIII_1$ and $XVIII_8$ induce a prolongation in PT only. PT was affected after 4 days of administration by compounds XII_1 and XII_2, while prolongation by all other compounds appeared after 48th hours. This means that the two former compounds have a slower action compared to, others. Furthermore, stope administration of the compounds for 3 days after the five successive doses revealed that the effect of the tested compounds III_1, III_6 and $XVIII_1$ had disappeared, while others were still acting. This might be explained on the basis of their short half-life while the other compounds have a prolonged one.

All the coagulation factors controlling values of PT have been reported to be synthesized in the liver and are vit.K dependent (Stephen *et al*., 1983). Oral anticoagulants including warfarin inhibit the biosynthesis of vit.k-dependent coagulation proteins (Rodegers, 1999). As the tested compounds have structural resemblance to warfarin, so increase in clotting times detected in this study could be attributed to their effect on the liver by competitively inhibiting vit.K in the enzyme reaction needed for the synthesis of these factors. Oral anticoagulants are antogonists of vit. K. Coagulation factors III, VII, IX and X; and the anticoagulant proteins C and S are synthesized mainly in the liver. They are biologically inactive unless certain glutamic acid residues (9 to 12 in number) are carboxylated by a microsomal enzyme system that utilizes reduced vitamin K as a cofactor. The modified γ-carboxylglulamyl (Gla) residues allow for $Ca^{2\pm}$ binding properties on these proteins that are essential for their assembly into an efficient catalytic complex (Goodman and Gillman's, 1991). The glutamic acid carboxylase probably acts deficiency or antagonism of vitamin K, decrease the rate of synthesis of the proteins.

Vitamin K epoxidise reduced vitamin k, and this reaction is some how coupled to carboxylase reaction. A number of different enzyme can reduce vitamin K *in vitro* to make it available for subsequent carboxylation reaction. The oral anticoagulants block the regeneration of reduced vitamin k and thereby induce a state of functional vitamin k deficiency (Suttie, 1987). Factor II, VII, IX and X are involved in the reaction of PT and APTT, so their decrease will increase PT and APTT. Prolonged administration of the compounds revealed the same pattern of effect on PT and APTT but to a lesser degree due to the smaller dose given to rats during prolonged administration. This was not the case with compound XII_3 which showed a more constant powerful effect with lower dose. This unexpected result may be due to the possible effect of XII_3 on the liver by competitive inhibition of vitamin k in the enzyme reaction needed for the synthesis of coagulation factors. A competitive inhibitor is usually a structural analogous of the substrate and binds to the enzyme at the substrate binding site but because it is not identical with the substrate, breakdown into products does not take place (Burtis and Ashwood, 1996). So the reaction shifts to the other side in high concentration of the inhibitor. At lower concentration the reaction shift forward and inhibition becomes faster.

Studding the effect of the tested compounds with prolonged use on liver and kidney functions as denoted by estimation of serum ALT, AST, BUN, and creatinine levels showed no changes in these parameters during all periods of the study. Although results of this work reveal that all tested compounds have anticoagulant activity and could be considered safe, compounds XII_2, XII_3, XII_{14} and III_{10} showed higher activity. Moreover compound XII_3 was found to be the most potent one even in lower doses, and by more investigations. It may be recruited to be a good oral anticoagulant drug.

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