International Journal
of
Pharmacy Review & Research
www.ijprr.com

e-ISSN: 2248 – 9207
Print ISSN: 2248 – 9193

ANTIOXIDANT AND ANTICOAGULANT ACTIVITY OF NOVEL N-SUBSTITUTED 4 METHYL 5,7 DI HYDROXYL COUMARIN AND ITS ESTER DERIVATIVES

[1]S.Venkataraman, [*]R.Meera, [1]Vidya Ramachandran, [2]P.Devi, [3]A.Aruna, [3]S.Padma Thanga Parameswari, [1]K.Nagarajan

[*1]Department of Pharmaceutical Chemistry, K.M.College of pharmacy, Uthangudi, Madurai –625107, Tamil Nadu, India.
[2]Department of Pharmacognosy, K.M.College of pharmacy, Uthangudi, Madurai –625107, Tamil Nadu, India.
[3]Department of Pharmaceutics, K.M.College of pharmacy, Uthangudi, Madurai –625107, Tamil Nadu, India.

ABSTRACT

Some novel N- Substituted 4 methyl 5,7 di hydroxyl coumarins were synthesized efficiently in two methods general synthesis and microwave method in 4 steps. Both the methods 12 compounds were synthesized. The synthesized compounds were identified by IR, NMR spectroscopic techniques. The present investigation deals with the synthesized compounds possessing good anti-oxidant and anti-coagulant activity. The anti-oxidant activity is carried out by DPPH method, Nitric oxide scavenging method and Hydrogen peroxide method. Compounds V and VI shown the maximum radical scavenging activity in DPPH method. Compounds V and II has shown the maximum activity in Hydrogen peroxide method. In Nitric oxide scavenging activity the compounds VI and VII have shown maximum activity. In *in vivo* Nitric oxide radical scavenging activity method the compounds effectively reduced the generation of nitric oxide from sodium nitroprusside by using Griess reagent. In anti-coagulant activity the Clotting time, bleeding time and Prothrombine time also observed. The compounds V and VI are more active in Clotting time. The compounds V and IV are more active in bleeding time. The compounds I and V are more active.

Key Words: Antioxidant activity, Anticoagulant activity, Novel N-Substituted 4 methyl 5,7 di hydroxyl coumarin derivatives.

INTRODUCTION

Coumarins and its derivatives have been proved as useful precursors for the synthesis of variety of medicinal agents [1]. The heterocycles derived from these have also been tested for their anti-HIV, anti-inflammatory, anti-convulsant, antioxidant, antibacterial, antifungal, anti-carcinogenic and anti- histaminic activities[2,3]. On the basis of our observation the present research work was carried out to synthesis some coumarin derivatives and further evaluate antioxidant and anti-coagulant activity. Antioxidant research is an important topic in the medical field as well as in the food industry. Recent researches with important bioactive compounds in many plant and food materials have received much attention. The oxidation induced by ROS can result in cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of many diseases, such as cancer, liver injury and cardiovascular disease [4-6]. Although the body possesses such defense mechanisms, as enzymes and antioxidant nutrients, which arrest the damaging properties of ROS continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond its capacity to control them, and cause irreversible oxidative damage. Therefore, antioxidants with free radical scavenging activities may have great relevance in the prevention and therapeutics of diseases in which oxidants or free radicals are implicated.

An agent that is used to prevent the formation of blood clots. Anticoagulants have various uses. Some are used for the prevention or treatment of disorders characterized by abnormal blood clots and emboli [7,8]. Anticoagulant drugs include intravenous heparin, which acts by inactivating thrombin and several other clotting factors that are required for a clot to form, and oral anticoagulants such as warfarin and dicumarol, which act

[*]**Corresponding Author R.Meera** E mail: meeraharsa23@gmail.com

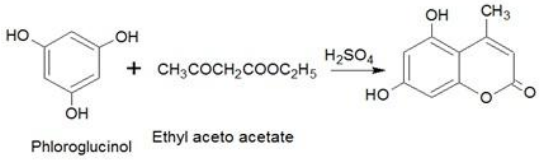
by inhibiting the liver's production of vitamin K dependent factors that are crucial to clotting. Anticoagulant solutions are also used for the preservation of stored whole blood and blood fractions and to keep laboratory blood specimens from clotting.

GENERAL SYNTHESIS

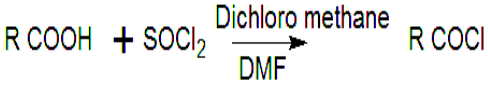
Step: 1 Synthesis Of 4-Methyl 5, 7 Dihydroxy Coumarin

Placed 100 ml of concentrated sulphuric acid in 500 ml 3 necked flask fitted with thermometer and dropping funnel. Immersed the flak in an ice bath, and the temperature was maintained below 10^0C. 0.1 mole of the Phloroglucinol in 0.103 mole of Ethyl acetoacetate was added drop wise with stirring. The temperature was maintained below 10^0C by means of ice salt bath during the addition (1-2 hours). The reaction mixture was kept at room temperature for 18 hours and then passed it with vigorous stirring into a mixture of 500 gm of crushed ice and 1 liter of water. The precipitate was collected by suction filtration and washed it three times with cold water. Dissolved the crude product of coumarin obtained in 5% of sodium hydroxide and checked the P.H. 10% HCl was added to this solution for the maximum precipitation to occur. Filtered and collected the pure recrystallized coumarin [9-12].

Step:2 Synthesis Of Acid Chloride

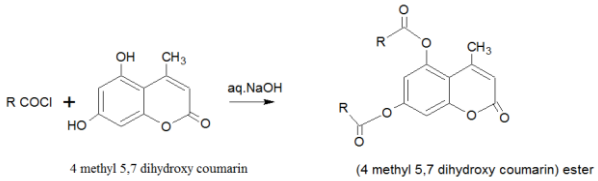


In a 250 ml distilling flask fitted with a separating funnel was placed 0.1 mole of the corresponding acid dissolved in 10-20 ml of dichloromethane. The side arm of this flask was attached to a long downward condenser, which in turn was connected by means of an adapter to a 500 ml filter flask. A calcium chloride drying tube was joined by short piece rubber tubing to the tubulature of the filter flask. While the flask containing the acid was heated on a water bath which was kept at 60^0C-65^0C, a 20 ml portion of freshly distilled thionyl chloride was gradually added from the separating funnel. Two to three drops of dimethyl formamide was added. The temperature of the water bath was maintained at 60^0C-65^0C for 2-3 hours until the vigorous evolution of gas had nearly ceased. As soon as the reaction had been completed the seperatory funnel was replaced by a thermometer and the excess thionyl chloride was carefully recovered by distillation. The acid chloride was next distilled over at 208-209^0C and was collected rejecting first few drops.



Step: 3 Synthesis of Coumarin Based Esters

Dissolved about 1.5 gm of crude coumarin obtained in step: 1 in 10-15 ml of 10% aqueous sodium hydroxide in a round bottom flask. To this solution, 2.4 equivalent of acid chloride was added at reduced temperature using an ice bath. The flask was shaken for half an hour and the content was filtered. The coumarin based ester prepared was recrystallised from ethanol.



MICROWAVE SYNTHESIS

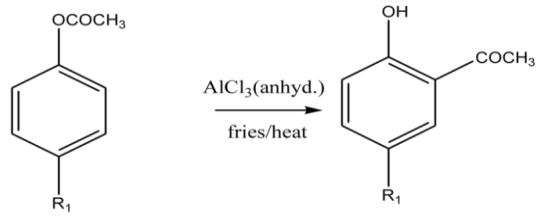
Step: 1 Procedure for Acetylation

A completely dried two necked round bottom flask was taken. Added substituted phenol (0.1 equivalents), acetic anhydride (0.4 equivalents) and pyridine (10 ml). The lid was closed and the flask was placed in an unmodified catalyst microwave oven at the power setting (Level 7, 80% power, 455 watts output) for 6 minutes. The TLC was checked after 6 minutes to ensure the completion of the reaction. After the completion of the reaction the reaction mixture was allowed to cool for some time. The cooled mixture was extracted with water: ethyl acetate mixture thrice. The organic layer was removed and some quantity of sodium sulphate was added to remove any traces of water and the solution was filtered in a dry round bottom flask. This organic layer was concentrated by distillation. Since the intermediate obtained was semisolid, it was not possible to find out the practical yield at this step.

Step: 2 Procedure For The Synthesis Of 2-Substitute Hydroxy Phenones

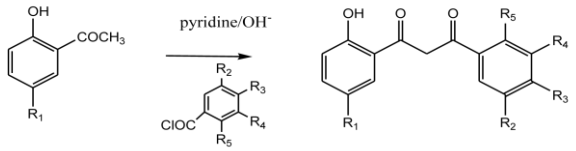
Removed a little quantity of the intermediate from step: 1 in a small vial and added small amount of ethyl acetate for TLC purpose. 0.1 equivalents of Aluminium chloride was taken into a beaker along with 15 ml CCL_4. The intermediate compound obtained after step: 1 was taken along with a little quantity of CCL_4 and this intermediate was added into a beaker containing Aluminium chloride drop wise with continuous stirring. The flask was kept in ice cold condition. After addition the reaction mixture was exposed to the microwave oven at the power settings (Level 3, 245 output watt) under mild intensity. The intensity was increased very slowly for power level 3 to 9 each for 4 minutes. The TLC was checked to ensure the completion of the reaction. After the completion of the reaction, the entire mixture was poured into a round bottom flask and the CCL_4 was distilled and cooled. The mixture of water: 6N HCL (50:50) was

prepared and the mixture was added very slowly into the cooled reaction mixture. The reaction mixture was extracted with Ethyl acetate thrice. The organic layer was removed in a dried beaker. Sodium sulphate was added into it and was filtered. The solvent was concentrated by distillation. The crude mixture of the product was obtained.



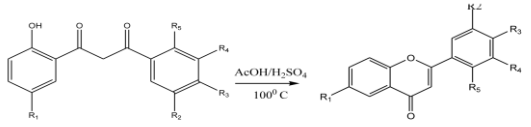
Step: 3 Incorporation of The Acid Chloride

The substituted/unsubstituted acid chloride (0.2 equivalents) was taken in 10 ml of pyridine into a round bottomed flask and added substituted or unsubstituted 2-hydroxyacetophenones obtained at the end of step: 2 into it. Powdered Sodium hydroxide (0.2 equivalents) was taken and added it to the above flask. Refluxed gently the reaction mixture in the microwave oven in the power setting (Level 4, 40% power, 280 watts) for 5 minutes. The completion of the reaction was checked by TLC. The reaction mixture was cooled and poured into excess of 6N HCL. Yellow precipitate was obtained. The precipitate was filtered, washed with water and dried. Recrystallized the crude material with ethanol.



Step: 4 Cyclization Reaction

The intermediate obtained at the end of step: 3 was taken along with acetic acid (10 ml) and concentrated Sulphuric acid in a beaker. Refluxed the reaction mixture in the microwave oven at the power settings (Level 8, 70% power. 490 watts) for 5 minutes. The completion of the reaction was ensured using TLC. The reaction mixture was cooled and poured into crushed ice. Precipitate was obtained. Filtered off the resulting precipitate, washed it with water and dried. Recrystallized with ethanol: water. The product was dried, weighed and the practical yield was noted.



Synthesised compounds

The synthisesed compounds are in general synthesis inScheme 1 derivative is4-methyl 5, 7 dihydroxycoumarin. In Scheme 2 derivativeToluene 4-sulphonyl chloride,3, 4 dimethoxy benzoyl chloride,4-chloro benzoyl chloride,Toluyl chloride,Benzoyl chloride**,** Acetyl chloride**,** 1-naphthyl acetyl chloride**,** P-methoxy benzoyl chloride. In Scheme 3 derivative4-methyl 2-oxo 2H chromene 5, 7 diylbis toluene 4- sulphonate compound I,4-methyl 2-oxo 2H chromene 5, 7 diylbis (dimethoxy benzoate) compound II,4-methyl 2-oxo 2H chromene 5, 7 diylbis (chloro benzoate) compound III, 4-methyl 2-oxo 2H chromene 5, 7 diylditoluate compound IV, 4-methyl 2-oxo 2H chromene 5, 7 diyldibenzoate compound V,4-methyl 2-oxo 2H chromene 5, 7 diyldiacetate compound VI,4-methyl 2-oxo 2H chromene 5, 7 diylbis (methoxy benzoate) compound VII,4-methyl 2-oxo 2H chromene 5, 7 diylbis (naphthyl acetate) compound VIII. In microwave synthesisderivatives of acid chlorides synthesized wereAcetyl chloride, Benzoyl chlorideSalicylyl chloride. In derivatives of Coumarin synthesized were1-benzo 2-phenyl pyran-3-one compound IX, 1-benzo 2-salicylyl pran-3-one compound X, 1-nitrobenzeno-2-phenyl pyran-3-one compound XI, 1-nitrobenzeno-2-phenyl Salicylyl pyran-3-one compound XII.

***IN VITRO* ANTIOXIDANT ACTIVITY**

DPPH radical scavenging activity

The free radical scavenging capacity of the test drugs were determined using DPPH. DPPH solution (0.004% w/v) was prepared in 95% methanol. Test drugs weremixed with95% methanol to prepare the stock solution (5 mg/mL). Freshly prepared DPPH solution (0.004% w/v) was taken in test tubes and the test drugs were added followed by serial dilutions (1 µg to 500 µg) to every test tube so that the final volume was 3mL and after 10 min, the absorbance was read at 515 nm using a spectrophotometer (HACH 4000 DU UV – visible spectrophotometer). Ascorbic acid was used as a reference standard and dissolve in distilled water to make the stock solution with the same concentration (5 mg/mL). Control sample was prepared containing the same volume without any drug and reference ascorbic acid. 95 % methanol was served as blank. % scavenging of the DPPH free radical was measured by using the following equation:

$$\%\text{scavenging Activity} = \frac{\text{Absorbance of the control} - \text{Absorbance of the test sample}}{\text{Absorbance of the control}} * 100$$

The inhibition curve was plotted for duplicate experiments and represented as % of mean Inhibition ± standard deviation. IC50 values were obtained by probit analysis.

Determination of total antioxidant capacity

The antioxidant activity of the drugs was evaluated by the phosphomolybdenum method. The assay is based on the reduction of Mo (VI)–Mo (V) by the drugs and subsequent formation of a green phosphate/Mo (V) complex at acid PH. A 0.3ml drugs were combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes

containing the reaction solution were incubated at 95^0C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer (HACH 4000 DU UV – visible spectrophotometer) against blank after cooling to room temperature. Methanol (0.3 ml) in the place of test drug is used as the blank. The antioxidant activity is expressed as the number of gram equivalents of ascorbic acid.

Scavenging of hydrogen peroxide

A solution of hydrogen peroxide (20mM) was prepared in phosphate buffered saline (PBS, PH 7.4). Various concentrations of 1 ml of the standard in methanol were added to 2 ml of hydrogen peroxide solution. The absorbance was measured after 10 min at 230 nm.

Nitric oxide radical inhibition

The test drug solutions and the standard ascorbic acid solutions were preparedin DMSO.

Preparation of the reagents

10mM solution of sodium nitroprusside wasprepared in distilled water.0.1g ofNEDD was dissolved in 60 ml of 50% glacial acetic acid by heatingand then diluted to 100 ml with distilled water.0.33 g of sulphanilicacid wasdissolved in 60 ml of 20% glacial acetic acid by heating and thendiluted to 100 ml with distilled water. The reaction mixture (6 ml)containing sodium nitroprusside (10mM, 4 ml), PBS (pH 7.4, 1 ml)and standard solution (1 ml) were incubated at room temperature for150 min. After incubation, 0.5 ml of the reaction mixture containingnitrite ion was removed, 1 ml of sulphanilic acid reagent was added,mixed well and allowed to stand for 5 min for completion ofdiazotization. Then, 1 ml of NEDD was added, mixed and allowed to stand for 30 min in diffused light. A pink colored chromophore wasformed. The absorbance was measured at 540 nm.

Scavenging of superoxide radical by alkaline DMSO method

To the reaction mixture containing 1 ml of alkaline DMSO (1 ml of DMSO containing 5 mMNaOH in 0.1 ml of water) and 0.3 ml of the standard (in DMSO), 0.1 ml of NBT (1 mg/ml) was added to give a final volume of 1.4 ml. The absorbance was measured at 560 nm.

***In vivo*antioxidant study in Animals**

Female wistar albino rats (100-150 g) were used for the studies. The animals were housed in large polypropylene cages in a temperature controlled room (37ºC±2ºC) and provided with standardized pellet feed and clean drinking water. The study protocol was duly approved by the Institutional Animal Ethical Committee.

Experimental design

After seven days of acclimatization, the rats were divided into four groups (n=6). Treatment was done for 8 days as follows:Group I is served as Control.Group II istoxic control received (CCl_4: olive oil (1:1) ;(0.7ml/kg.) at every 72 h.Group III is received standard drug (50 mg/kg/day) for 7 days and simultaneously administered CCl_4 at every 72 h.Group IV is received test compound (250 mg/kg/day) for 7 days and simultaneously administered CCl_4 at every 72 hr.Group V is received test compound (500 mg/kg/day) for 7 days and simultaneously administered CCl4 at every 72 h. After 24 h of the last dose, all the animals were anaesthetized with ether and sacrificed. The blood was collected from retro-orbital plexus. The blood samples were allowed to clot and the serum was separated by centrifugation at 2500 rpm for 15 min at 37° C and the serum was used for biochemical estimation. All the animals were then sacrificed and liver tissues were collected for the evaluation of *in vivo* antioxidant studies.

Nitric oxide radical scavenging activity

Test compound effectively reduced the generation of nitric oxide from sodium nitroprusside. Scavenging of nitric oxide radical is based on the generation of nitric oxide. Sodium nitroprusside in buffered saline, which reacts with oxygen to produce nitrite ions that can be measured by using Griess reagent. The absorbance of the chromophore is measured at 546 nm in the presence of the fractions [13-19].

ANTICOAGULANT ACTIVITY[19]

Animals

Adult male Wistar rats (200 to 300 g) were used. Animals were distributed among groups according to the balanced, Latin-square block design based on body weight. Room temperature was kept constant (20-22°C) with 12-12 h light-dark cycles and food and water ad libitum.

Blood coagulation method (CT)

The tail of the animal was warmed for 1 min in water at 40°C. The tail was dried and cut at the tip with a razor blade. A 25 µl sample of capillary blood was collected into a microhematocrit glass capillary. The chronometer was started when the blood first made contact with the glass capillary tube. The blood was left to flow by gravity between the two marks of the tube, 45 mm apart, by tilting the capillary tube alternately to +60° and -60° angles with respect to the horizontal plane until blood ceased to flow (reaction end point).

Bleeding time method (BT)

The tail of the rat was warmed for 1 min in water at 40°C and then dried. A small cut was made in the middle of the tail with a scalpel. Bleeding time started when the first drop touched the circular filter paper. It was checked at 30s intervals until bleeding stopped.

Animal blood collection

For the remaining blood coagulation variables, animals were anaesthetized with chloral hydrate (4% solution, 7 ml/kg) prior to blood withdrawal. Arterial blood was collected by aspiration from the iliac bifurcation, which provided an abundant sample free of

hemolysis. The blood sample was immediately emptied into a plastic tube containing 0.11 M sodium citrate at a ratio of 1:10 anticoagulant blood, gently mixed and centrifuged at 2500 rpm at 4°C for 10 min. Plasma was separated and maintained in ice bath throughout its processing.

RESULTS AND DISCUSSION

Antioxidant Activity

DPPH is usually used as a substrate to evaluate antioxidant activity. DPPH assay is based on the measurement of the scavenging ability of antioxidant towards the stable DPPH radical. The DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activity of the test compounds was given in the table. DPPH antioxidant assay is based on the ability of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance.

Total antioxidant capacity of the compounds, expressed as the number of gram equivalents of ascorbic acid. The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm. Compounds V and VI has shown the maximum radical scavenging activity.

Scavenging of hydrogen peroxide

Hydrogen peroxide is generated *in vivo*by several oxidase enzymes. In this method, when an antioxidant is incubated with hydrogen peroxide, the decay or loss of hydrogen peroxide is measured spectrophotometrically. Hydrogen peroxide is a weak oxidizing agent which inactivates enzymes by oxidation of the essential thiol (SH-) groups. It rapidly transverses cell membranes and once inside the cell interior, interacts with Fe^{2+} and Cu^{2+} to form hydroxyl radicals, which is harmful to the cell. Compounds V and II has shown the maximum hydrogen peroxide scavenging activity.

Nitric oxide radical inhibition

Nitric oxide is implicated in diseases such as cancer and inflammation. It also mediates smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated cytotoxicity. Sodium nitroprusside spontaneously generates nitric oxide at physiological pH, in aqueous solutions. The nitric oxide, generated is converted into nitric and nitrous acid on contact with dissolved oxygen and water. The liberated nitrous acid was estimated using a modified Griess-Illosvoy method. Nitrous acid reacts with Griess reagent, to form a purple azo dye. In the presence of antioxidants, the amount of nitrous acid will decrease and the degree of decrease in the formation of purple azo dye will reflect the extent of scavenging. Compounds VI and VII has shown the maximum nitric oxide scavenging activity of 135.2±4.175 and 153.7±4.145 respectively.

In the case of *in vivo* Nitric oxide radical scavenging activity

Test compounds effectively reduced the generation of nitric oxide from sodium nitroprusside. Scavenging of nitric oxide radical is based on the generation of nitric oxide. Sodium nitroprusside in buffered saline, which reacts with oxygen to produce nitrite ions that can be measured by using Griess reagent. The absorbance of the chromophore is measured at 546 nm in the presence of the fractions.

ANTICOAGULANT ACTIVITY

Clotting Time

The compounds V and VI are more active

Bleeding Time

The compounds V and IV are more active .

Prothrombin Time

The compounds I and V are more active.

Anti oxidant parameters

Table 1. Radical scavenging activity- *in vitro* parameters

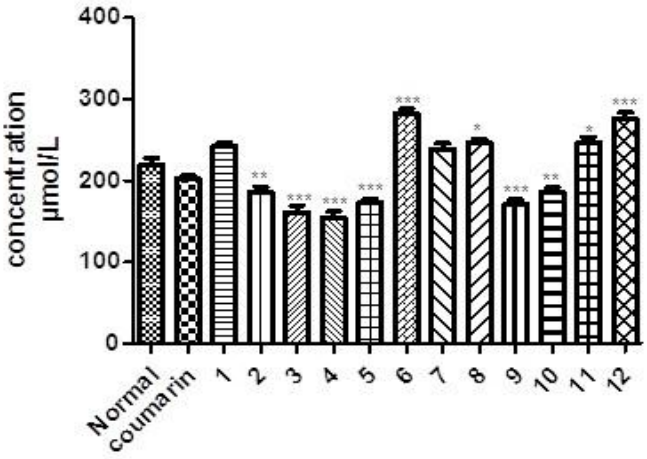
Groups	Nitric oxide	H_2O_2	DPPH
1	172.0±4.412	301.3±5.925	13.33±0.8819
2	139.7±3.648	145.3±2.155	6.167±1.302
3	137.3±4.030	398.2±6.400	9.333±0.6146
4	132.0±3.011	311.5±4.161	16.83±0.9732
5	154.2±8.207	119.3±2.186	2.333±0.3333
6	83.17±1.078	162.7±2.028	3.833±0.3073
7	135.2±4.175	151.8±1.922	7.833±0.4773
8	153.7±4.145	154.7±2.906	14.50±1.057
9	184.3±4.169	175.2±2.330	14.50±0.9916
10	170.0±6.957	204.5±2.045	24.17±1.014
11	144.0±3.661	214.8±1.682	17.83±0.4773
12	143.7±3.127	236.3±4.326	16.00±0.8944
Ascorbic acid	93.00±0.577	193.0±1.807	2.115±0.4618

***IN VIVO*- anti oxidant parameters**

Table 2. Anti-oxidant activity (Nitric oxide)

Groups	Concentration µmol/L
Normal	219.2±8.183
Coumarin	202.8±3.655
1	241.7±5.578
2	185.0±7.638**
3	160.7±8.381***
4	154.5±7.182***
5	172.7±4.842***
6	281.7±6.667***
7	238.5±7.140
8	246.7±4.379*
9	170.7±5.554***
10	186.2±4.963**
11	246.0±7.638*
12	275.7±7.342.***

Figure 1. Anti-oxidant activity (Nitric oxide)



Haemostatic parameters

PT determination For Prothrombin determination thromboplastins were used, from rabbit brain

Table 3. Clotting Time

Groups	Clotting time
Normal	130.5±3.481
Coumarin	153.3±3.201***
1	142.8±4.840
2	144.8±2.561*
3	134.2±2.822
4	148.0±3.204**
5	172.3±2.565***
6	140.8±5.275
7	170.8±2.626***
8	129.8±2.040
9	139.2±3.400
10	146.3±2.290*
11	146.5±2.975*
12	142.8±4.061

Figure 2. Clotting Time

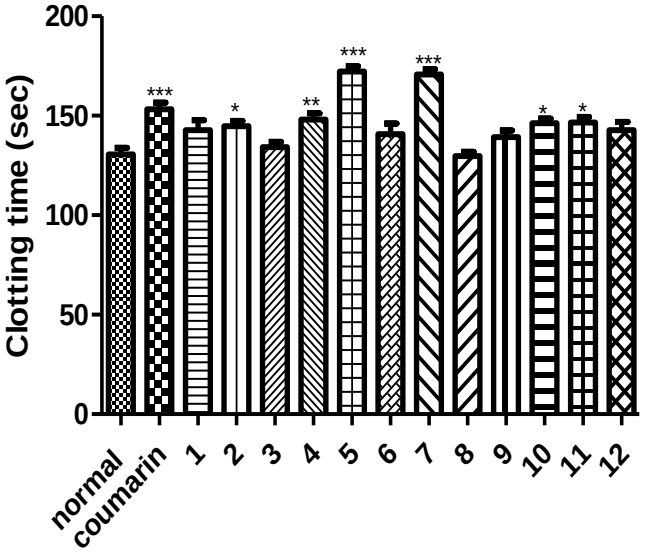


Table 4. Prothrombin Time

Groups	Prothrombin time (sec)
Normal	24.17±0.9458
Coumarin	43.00±1.366***
1	51.00±2.490***
2	32.33±1.606**
3	24.33±1.145
4	18.50±0.7638
5	47.83±2.386***
6	44.50±1.232***
7	33.67±0.8819***
8	21.17±0.8724
9	24.00±1.155
10	35.00±1.155***
11	24.33±1.453
12	28.17±1.302

Figure 3. Prothrombin Time

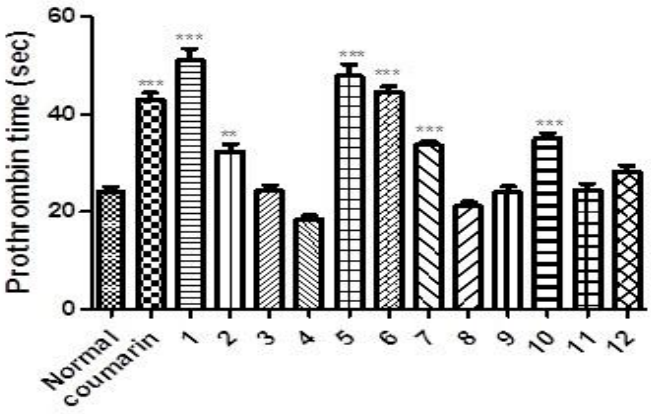


Table 5. Bleeding Time

Groups	Bleeding Time(sec)
Normal	85.33±1.229
Coumarin	101.7±2.871***
1	80.17±1.973
2	85.33±1.994
3	94.67±1.116*
4	111.3±2.390***
5	124.2±1.600***
6	105.3±1.856***
7	98.67±3.685***
8	100.7±2.186
9	93.50±1.335
10	90.67±2.290
11	94.67±1.726*
12	103.2±2.774***

Figure 4. Bleeding Time

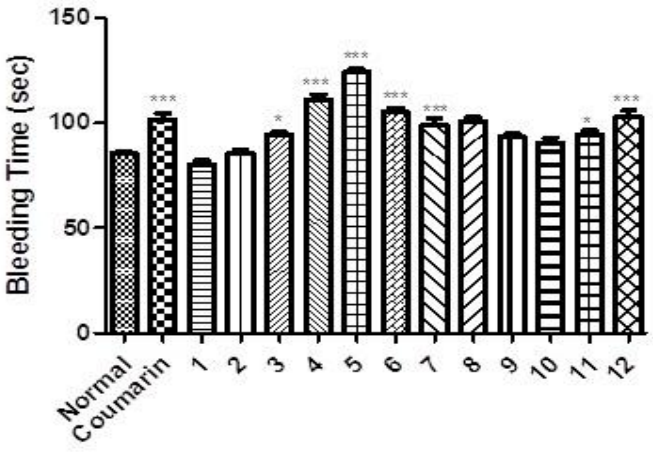


Figure 5. Graph for IC_{50} nitric oxide

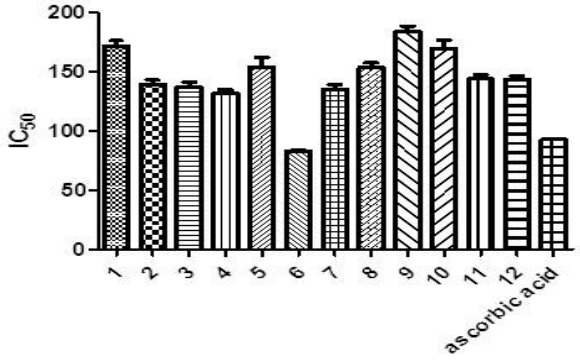


Figure 6. Graph for H2O2

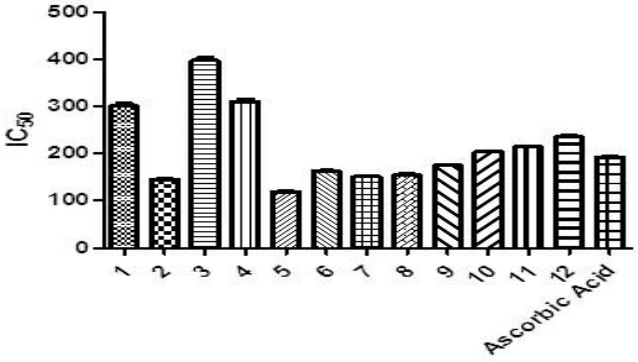


Figure 7. Graph for DPPH assay

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