# Aquatic Plants from Minnesota Part 2 - Toxicity, Anti-Neoplastic, and Coagulant Effects

by

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FOREWORD

This Bulletin is published in furtherance of the purposes of the Water Resources Research Act of 1964. The purpose of the Act is to stimulate, sponsor, provide for, and supplement present programs for the conduct of research, investigations, experiments, and the training of scientists in the field of water and resources which affect water. The Act is promoting a more adequate national program of water resources research by furnishing financial assistance to non-Federal research.

The Act provides for establishment of Water Resources Research Centers at Universities throughout the Nation. On September 1, 1964, a Water Resources Research Center was established in the Graduate School as an interdisciplinary component of the University of Minnesota. The Center has the responsibility for unifying and stimulating University water resources research through the administration of funds covered in the Act and made available by other sources; coordinating University research with water resources programs of local, State and Federal agencies and private organizations throughout the State; and assisting in training additional scientists for work in the field of water resources through research.

This Bulletin is number 47 in a series of publications designed to present information bearing on water resources research in Minnesota and the results of some of the research sponsored by the Center. The Bulletin is concerned with the study of the toxicity, antineoplastic, coagulant and anticoagulant effects of 22 Minnesotan aquatic plants. The study is part of a research project aimed at finding a nutritional, medicinal or industrial use for the unwanted aquatic plants in lake shoreline areas. It is possible that some aquatic plants may contain industrially useful gums-mucilages, or new useful antimicrobial, anticoagulant, or antineoplastic therapeutic principles. If a good industrial, medicinal, or nutritional use for aquatic plants can be discovered, the results of the research could provide an economic incentive for aquatic plant collection and control. The successful completion of the project may significantly assist the State and Nation in partially solving their lake pollution problems.

This Bulletin is related to the following research project:

OWRR Project No.: A-025-Minn.

Project Title: Alleviation of Lake Pollution by Utilization of Aquatic Plants for Nutritional, Medicinal or Industrial Purposes

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Publication Abstract:

In this study, toxicity, antineoplastic, coagulant and anticoagulant effects of the following 22 Minnesotan aquatic plants were evaluated in terms of pharmacological properties: Anacharis canadensis, Calla Polustris, Carex lacustris, Ceratophyllum demersum, Chara vulgaris, Eleocharis smallii, Lemna minor, Myriophyllum exalbescens, Nuphar variegatum, Nymphaea tuberosa, Potamogeton amplifolius, P. natans, P. pectinatus, P. richardsonu, P.

zosteriformis, Sagittaria cuneata, S. latifolia, Sparganium eurycarpum, S. fluctuans, Typha angustifolia, Vallisneria americana, and Zizania aquetica. Toxicity of skellysolve F, chloroform, 80% ethanol and water extracts of these aquatic plants were evaluated in a number of animal experiments in Swiss Webster mice. Antineoplastic experiments involved amelanoma tumor cells. In vivo prothrombin time (PT) and partial thromboplastin time (PTT) were assayed in anti-coagulation experiments.

The toxicity of the aquatic plants in general was found to be relatively low. The $LD_{50}$ for the most toxic one, i.e., N. tuberosa (stem), in mice was 3 gm of dry plant material/kg (ca. 25.4 gm of wet plant material/kg). Only Nuphar variegatum indicated an anticancer possibility, the remaining aquatic plants had no significant inhibition activity at the doses selected. Normal partial thromboplastin time for mice was 51 seconds and only the prolongation of PTT (longer than 61 seconds) was observed in 50% of the aquatic plants tested. Among these plants, the most significant increase of PTT (more than 20 minutes) was observed in Carex lacustris, Myriophyllum exalbescens, Nuphar variegatum and Nymphaea tuberosa.

Publication Descriptors: *Aquatic Plants/ *Pharmacological Properties/ *Minnesota/ Toxicity/ Antineoplastic Activity/ Coagulants/ Anti-Coagulants/ Cancer

Publication Identifiers: *Prothrombin Time/ *Partial Thromboplastin Time/ Anticancer



# I. INTRODUCTION

## A. Scope of the Study

The collection, identification and survey of the chemical constituents of 22 Minnesotan aquatic plants have been reported (1). In this study, toxicity, antineoplastic, coagulant and anticoagulant effects of these aquatic plants were evaluated.

## B. Pharmacological Screenings

Pharmacological screening serves to demonstrate whether the plant extracts are worthy of further attention and to indicate which among them have the most interesting pharmacological properties. Pharmacological inertness of a certain plant is quite important to avoid duplicate negative efforts by other workers.

### 1. Toxicity

An indication of toxicity for certain plant material is usually an evidence for the presence of biologically active material in that particular plant. Since "toxic" and "physiologically active" terms are relative, proper dose administration of the plant extracts in question may discover a pharmacological activity worthy of further study. Experiments involved a $LD_{50}$ (leathal dose causing 50% death of experimental animals) determination should be as uniform as possible involving animals distributed in a random order, tested on the same day by the same person, and under as nearly identical environmental conditions as possible. It is impossible to measure directly the dose needed to cause 50% death of tested animals. Instead, a series of doses are given, the corresponding results are measured, and the $LD_{50}$ value is obtained by interpolation. If each dose is given to a large number of animals and the mean responses plotted against doses, a dose-response curve will result. Plotting the logarithm of the doses, preferably in three doses in geometric progression results in a straight line (2-4). The number of animals used for each dose level is determined by what degree of probability the investigator is willing to accept.

The pharmacological effect of the alkaloid nupharine from Nuphar luteum was investigated by Domitrov, in 1964 (5,6). The $LD_{50}$ for albino mice was 112 mg/kg subcuteneously and 31 mg/kg intravenously. Nupharine had a spasmolytic action on isolated rabbit intestine. It caused a 2-3.4°C drop of body temperature five hours after intravenous injection, and stimulated respiration.

The toxicity of another alkaloid, nupharidine, from Nuphar luteum was also studied (5). The $LD_{50}$ for albino mice was 97 mg/kg subcuteneously and 29 mg/kg intravenously. It stimulated the motor activity and raised the tone of the smooth intestinal muscles and blood vessels in albino mice. The alkaloid also had a "delayed" heart action in rabbits, and a positive inotropic effect on isolated mammalian hearts.

The lupine alkaloid, deoxynupharidine, from Nuphar japonicum strongly depressed respiration and caused respiratory failure at a dosage of 8-15 mg/kg in rats. This alkaloid also had an antiacetylcholine, an antihistaminic, and hypertensive activities by causing constriction of peripheral blood vessels with a subsequent increase in the blood pressure (7).

Odintsova (8) studied the plant Nymphaea alba (white water lily), and observed that the toxicity was quite low. Dogs could tolerate 100 ml of a 6% extract three times a day for 45 days. The plant extract exhibited a "hypotensive effect on isolated frog's heart". A 1:100,000 infusion intensified heart contractions, whereas a higher infusion concentration exerted an opposite effect. In rabbits, the infusion slightly raised the blood pressure, but in dogs and rabbits that had an experimental pituitrin hypertension, it exerted a distinct hypotensive effect.

2. Antineoplastic activity

The application of plants in the treatment of cancer is ancient and has been ascribed by primitive people (9). Interest in higher plants as a potential source of tumor-inhibiting agents probably began with colchicine, an alkaloid derived from the autumn crocus (10). More recently, podophyllotoxin derived from Podophyllum peltatum was shown to inhibit some experimental tumors (11), vincaleukoblastine (12,13) and leurocristine (14) from Catharanthus roseus have been used clinically to control certain tumors, and camptothecin (15,16,17) from Camptotheca acuminata has shown to exhibit potent antileukemic and antitumor activities in animals. The active anti-cancer principles vary greatly in their chemical structures. Farnsworth (1) listed the tumor inhibitors isolated and characterized up to 1966, as alkaloids (vincaleukoblastine), cardenolides (apocannoside), lignans (podophyllotoxin), flavonoids (eupatorin), tannins (Rumex hymenosepalus), proteins (Gutierrezia sarothrae), sesquiterpene lactones (gallardin), tetracyclic triterpenes (elatericin A), etc. Since then many more alkaloids (18-22), lignan (23) and saponin (24) antitumor agents have been described.

Tumor screening experiments are carried out continuously by the Cancer Chemotherapy National Service Center (CCNSC) screening laboratories using established protocols (25). This Center has screened more than 26,000 plant species and found that 10% were active against one or more tumors tested (26). The University of Texas screened plant extracts for antitumor effects in egg-cultivated tumor tissue and mouse-grown tumors (27-36). The tissue culture technique has served as a valuable method for the screening of agents for their possible effect against tumors. A differential action on normal and malignant cells is observed at the cellular level, however, the normal cell line may lack certain immune component. A simple, rapid and relatively inexpensive tissue culture cytotoxicity test suitable for large scale cancer chemotherapy screening has been described by Toplin (37). The cylinder plate (38) and agar diffusion (39) methods had been applied as well for the determination of anti-tumorigenic action on the cells of Ehrlich's ascites. Many anticancer screening procedures have been reported (40-52).



3. Coagulants and anti-coagulants

a. Coagulation mechanism

The mechanism of the spontaneous change from fluid blood to solid clot has been a subject of interest for years. Many workers have produced many different theories and, unfortunately, many different terminologies. An important advance has been the introduction of a standard nomenclature which assigns a Roman numeral to each clotting factor (Fig. 1).

Synonym for clotting factors:

| | | |
|---|---|---|
| Factor I | : | Fibrinogen |
| Factor II | : | Prothrombin |
| Factor III | : | Thromboplastin |
| Factor IV | : | Calcium |
| Factor V | : | Labile Factor, Plasma Accelerator Globulin, Proaccelerin |
| Factor VII | : | Proconvertin |
| Factor VIII | : | Antihemophilic Globulin, Antihemophilic Factor |
| Factor IX | : | Christmas Factor, Plasma Thromboplastin Component, Antihemophilic Factor B |
| Factor X | : | Stuart-Prower Factor |
| Factor XI | : | Plasma Thromboplastin Antecedent |
| Factor XII | : | Hageman Factor |
| Factor XIII | : | Fibrin Stabilizing Factor, FSF |

b. Anti-coagulants

Anticoagulants are substances of drugs which delay coagulation of blood. There are three general types (54) used in the clinic:

1) Calcium sequestering agents. Calcium is essential to several steps in the clotting process, hence its removal prevents clotting. They are employed only in withdrawn blood. Examples are sodium citrate or sodium oxalate.

2) Heparin and heparin substitutes. These agents interfere with clotting by inhibiting the conversion of prothrombin to thrombin, the action of thrombin on fibrinogen, and the rupture of blood platelets.

3) Prothrombopenic anti-coagulants. They competitively inhibit vitamin K in the hepatic production of prothrombinogen, the immediate precursor of plasma prothrombin and impair the blood coagulation. Examples are sodium warfarin, coumarin and its derivatives.

The carrageenans, which occur naturally in the red seaweeds, constitute a closely related group of sulphated galactans, and have been claimed to have an anti-coagulant power equal to that of heparin (55). Cinchona alkaloids have been shown to potentiate oral anti-coagulants by depressing the prothrombin complex and decreasing the synthesis of vitamin K (56). Continuous use of cathartics, such as mineral oils, may decrease the absorption of vitamin K (57). Bromelains and papain may potentiate oral anti-coagulants (58). Onions may enchance fibrinolytic activity, although this effect is transient (59). Finally, anti-coagulant activity has been observed in many coumarin derivatives found in nature (60).

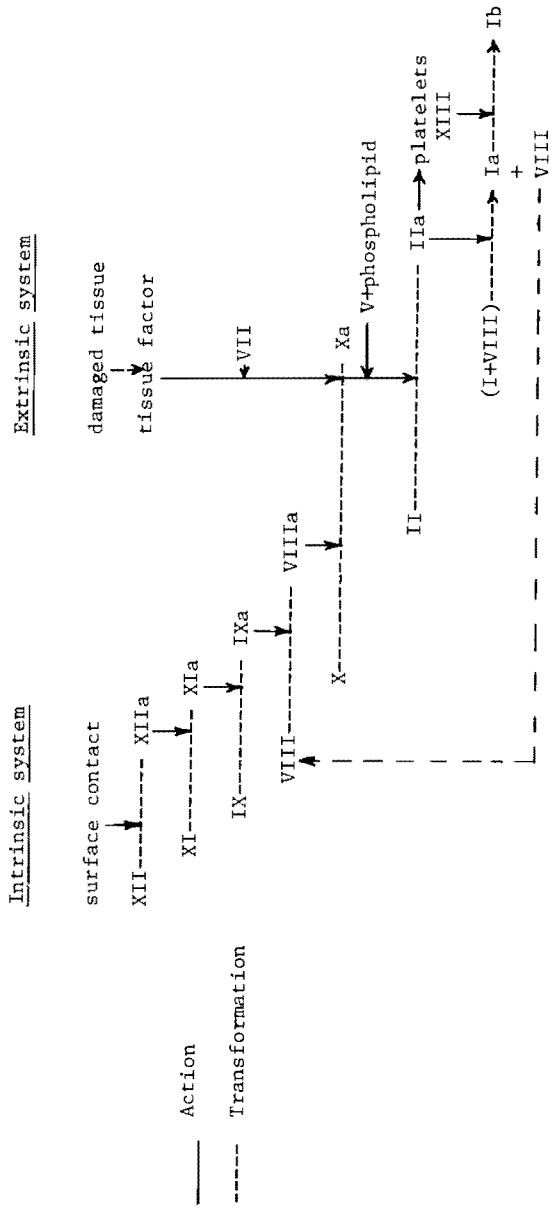


Figure 1. Interactions of the clotting factors (53).



c. Techniques in the blood coagulation studies

Clinical detection of blood disorders and the monitoring during the course of long-term heparin therapy have prompted the development of various coagulation techniques, such as the determination of recalcification time (61), thrombin time (62), whole blood clotting time (63), activated whole blood coagulation time (64), prothrombin time (65) and partial thromboplastin time (66-68). The latter two methods are found to be more sensitive and reproducible, and will be discussed in detail.

1) Prothrombin time (PT)

The principle involved is based on the fact that thromboplastin (tissue extract) activates Factor VII, which in turn activates S, V and II. Factor II (Prothrombin) is activated to thrombin which converts fibrinogen to a fibrin clot (this is also called the extrinsic system). The procedure consists of adding thromboplastin reagent and calcium to citrated plasma and recording the clotting time. Rabbit or human brain (acetone extracted) lipoprotein is usually used to supply the thromboplastin. Other tissues can be used but are more difficult to keep blood-free and may be contaminated with other factors.

Since anti-coagulants are detoxified in the liver, substances altering hepatic function may interfere with the formation of coagulation factors and lead to an increased prothrombin time (69,70).

2) Partial thromboplastin time (PTT)

Partial thromboplastin time may be defined as the clotting time of recalcified plasma in the presence of a partial (phospholipid) factor rather than the complete thromboplastin (protein and phospholipid) used in the prothrombin time test. The technique for the performance of partial thromboplastin time tests is similar to that for the prothrombin time. The commonly used partial thromboplastins are extracts of brain tissue (cephalin) and soy bean extracts (inositin).

PTT is prolonged by deficiencies of any of the plasma clotting factors in the intrinsic blood clotting system, and by anti-coagulants (e.g., heparin and fibrinogen degradation products). PTT will also be prolonged by moderate deficiencies (less than 40 to 50% of the normal mean) of Factors V, VIII, IX, X, XI and XII, and severe deficiencies of prothrombin (Factor II) and fibrinogen. High levels of any clotting factor in the intrinsic sequence may shorten the PTT. The commonest situation encountered is a high level of Factor VIII producing a short PTT. The PTT is not affected by Factor VII levels, as Factor VII is not involved in the intrinsic clotting system.

Factor XII is the first factor in the intrinsic sequence. It is activated by contact with wettable surfaces. When PTT is performed in a glass tube without an activating agent, the activation of Factor XII by the wall of the glass tube is incomplete and variable from tube to tube. In order to circumvent this problem, the "activated" PTT was devised. In

the kaolin PTT, a suspension of kaolin powder is mixed with the phospholipid suspension. Kaolin provides a huge surface area for maximal activation of Factor XII and the assay much improved.

d. General considerations

In a system where so many factors are responsible for the end product, it is imperative that working conditions and technique be of the highest caliber in order to get reproducible values. When glassware is used it must be extremely clean. Disposible plastic equipment, where applicable, is preferred to ensure cleanness and absence of any contact activation factor. Blood sample should be free of any foreign substance, such as tissue juices which may initiate clotting and false result will occur. In addition, care must be exercised to ensure that the ratio of blood to anti-coagulant is kept constant, since too much anti-coagulant is as undesirable as too little anti-coagulant. Plasma should be separated from the cells as quickly as possible, no more than 30 minutes after blood is drawn, and it should be kept at 4°C until tested. Temperature must be constant. Most coagulation tests are performed at 37°C and variation should not be more than $\pm$ 0.5°C. Distilled water used should be free of heavy metals. A constant optimum centrifugal force should be used.

## II. MATERIALS AND METHODS

### A. Plant Materials

Skellysolve F, chloroform, 80% and 50% ethanol, acidic and basic water extracts of the following plant species were used (those in parentheses are the abbreviations used in the Tables): Anacharis canadensis (Ac), Calla palustris (Cp), Carex lacustris (Cl), Ceratophyllum demersum (Cd), Chara vulgaris (Cv), Eleocharis smallii (Es), Lemna minor (Lm), Myriophyllum exalbescens (Me), Nuphar variegatum (Nv), Nymphaea tuberosa (Nt), potamogeton amplifolius (Pa), P. natans (Pn), P. pectinatus (Pp), P. richardsonii (Pr), P. zosteriformis (Pz), Sagittaria cuneata (Sc), S. latifolia (Sl), Sparganium eurycarpum (Se), S. fluctuans (Sf), Typha angustifolia (Ta), Vallisneria americana (Va) and Zizania aquatica (Za). The collection, identification and extraction procedures were previously reported (1).

### B. Toxicity and $LD_{50}$ Determination

1. General survey of toxicity in aquatic plants

Toxicity of skellysolve F, chloroform, 80% ethanol and water extracts of aquatic plants collected were evaluated in a number of animal experiments in Swiss Webster mice. Total numbers of death were recorded for comparison.



2. $LD_{50}$ determination

Male Swiss Webster mice weighing 20-25 gm were kept in an animal room with a constant temperature of 27°C. Regular laboratory chow and water were given ad lib. Ethyl alcohol (50%) extracts were prepared from C. demersum and N. tuberosa and 0.5% sodium carboxymethyl cellulose was used as the suspending agent in the preparation of proper dose levels (ef. Table 2). Sample doses (0.3 ml in 0.1 ml increment) in an arithmatic series were injected intraperitoneally (I.P.) into the mice. A total of 40 mice and three dose levels were used for each $LD_{50}$ determination (10 mice per dose level). Time of injection, signs displayed and the time of death were recorded. The observation period for the incidence of death was 72 hours. Sodium carboxymethyl cellulose (0.5%) was used as the control. Bliss (3) statistical method of probit vs. log-dose graph (Fig. 2) was used for the calculation of $LD_{50}$ values.

### C. Antineoplastic

1. Hamster tumor screening

a. Tumor and host animal

Amelanoma tumor cells were supplied by Dr. Henry Smith (Gleenwood Hill Hospital, Minneapolis, Minn.). The tumor was first discovered as a spontaneous malignant melanoma in hamsters by Fortner (71) and was transformed into an amelanoma strain by the tissue culture method. The host animals used in this experiment were golden Syrian hamsters.

b. Transplantation of tumor

Intramuscular implantation was used to transfer the malignancy to 40-49 days old male golden Syrian hamsters each weighing 80-95 gm. The transplantation procedure was carried out semi-aseptically as follows: The solid tumor was removed with sterilized surgical equipment from a sacrificed hamster and transferred to a sterile Petri dish containing 15 ml of normal saline. The tumor was chopped into small pieces with sterile scissors, and 1 ml of the cell suspension injected with a 19 gauge needle syringe intramuscularly into the hamster.

c. Test procedures

Three samples were prepared for testing from each plant. Equal volumes of skellysolve F and chloroform extracts were evaporated to dryness and reconstituted in 2% methyl cellulose solution to a concentration of 100 mg of total extract/ml. Ethyl alcohol extracts (80%) were freed of ethanol by heating in a water bath (70°C), and used in a concentration equivalent to 3.75 gm of dried plant material/ml. Equal volumes of acidic and basic water extracts were combined and tested in the concentration equivalent to 3.75 gm of dried plant material/ml.

Each extract was administered intramuscularly, 1 ml each time, on the day before and on the day of tumor transplantation. If a lethal result was

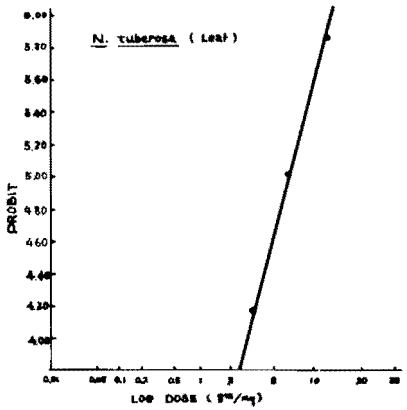


Figure 2. Probit vs. log-dose graph for the $LD_{50}$ determination of the 50% ethanol extract of N. tuberosa (leaf).



observed, the dosage was decreased in half until a tolerated dose was established.

Tumor inhibition was observed by a comparison of the lag period (days required to detect the appearance of the solid tumor, cf Fig. 3), and by a comparison of the tumor weight at the end of 15 days for both control and treated hamsters (Fig. 4). All extracts which showed a tumor weight inhibition of 50% or more and a prolongation of two or more days of lag period were considered as being significant and were each repeated on 4 hamsters.

2. Cancer Chemotherapy National Service Center (CCNSC) Screening

Ethanol (50%) plant extracts which had not been screened by CCNSC were submitted to Dr. L.J. Hartwell for anticancer screening under the CCNSC standard protocol (72).

D. Anti-coagulation

1. Samples and standard

In vivo prothrombin time (PT) and partial thromboplastin time (PTT) were assayed on male Swiss Webster mice.

Equal volumes of skellysolve F and chloroform extracts were combined, freed of solvents, and suspended in 2% methyl cellulose to make 100 mg of dried extract/ml. Also studied were 80% ethanol extracts filtered through a Swinnex-47 millipore filter. Dicoumarol (10 mg/ml) was used as the standard.

2. Test procedures

a. Preparation of blood samples

The test was performed by a modification of the method originally described by Langdell, Wagner and Brinkhours (66).

Male Swiss Webster mice (20-25 gm) were maintained at all times on an unrestricted diet of laboratory food and water. Two intraperitoneal injections of each sample were made, the first at 24 hours and the second at 12 hours before the withdrawal of blood. The blood (0.5 ml) was withdrawn from etherified treated and control mice (0.9% NaCl used in place of extracts) by cardiac puncture with a 19 gauge needle. Any slow flow of blood due to the imperfect puncture was repeated. The blood was transferred immediately into plastic tubes containing 0.1 ml of 0.1 M sodium citrate solution. The tubes were centrifuged in a pre-refrigerated table top model centrifuge (International Equipment Co., Neldham His., Mass., Model CL) at speed 7 for 10 minutes. The plasma was aspirated carefully with a disposable pipette from the red blood cells and used immediately for PT and PTT tests or stored in crushed ice until needed.

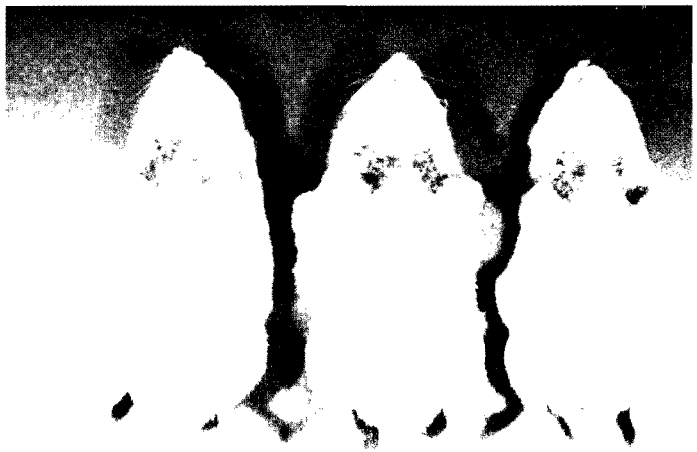

Figure 3. The appearance of amelanoma tumor in golden Syrian hamsters. (1 - Control; 2, 3 - Treated with the skellysolve F and chloroform combined extract of N. variegatum).

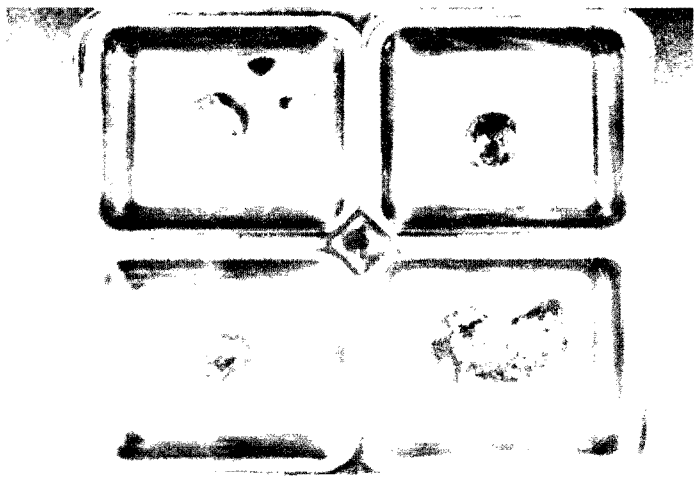

Figure 4. Isolated amelanoma tumor from golden Syrian hamsters (1 - Treated with skellysolve F and chloroform combined extract of N. variegatum; 2 - Control).



b. Determination of prothrombin time (PT) and partial thromboplastin time (PTT)

The reagents used were: Verify normal citrate (General Diagnostic Division, Warner-Chilcott Lab., Morris Plains, N.J.), the product is the pooled, freeze-dried normal human plasma standardized to contain optimal concentrations of clotting factors; Activated thromboplastin with $CaCl_2$ (Dade Coagulation Products), the produce is a stabilized suspension of the thromboplastic components extracted from acetone-dehydrated rabbit brain; Activated cephaloplastin (Dade Coagulation Products, Miami, Fla.), the product is an extract of rabbit brain, prepared according to the method of Bell and Alton (73), with a plasma activator added. It is standardized in the liquid form to provide a reliable platelet factor substitute; 0.1 M sodium citrate and 0.02 M calcium chloride.

The vials containing activated thromboplastin for the PT test, or activated cephaloplastin in case of PTT test, were gently inverted once or twice to suspend the particles. A 0.1 volume of either activated thromboplastin or cephaloplastin was placed in a 7.5 x 75 mm plastic disposable test tube in the 37°C water bath for one minute, and then 0.1 ml of treated plasma was added for an additional minute. Pre-warmed 0.02 M $CaCl_2$ (0.1 ml) was rapidly added to the mixture, and the zero time recorded. The mixture in the tube was gently tilted about once per second and was held against at adequate light source until the end-point, a gel-like fibrin clot, was observed. Standarized plasmas were run concurrently with the treated plasma to insure the quality of the reagents used. Triplicate determinations were done for each sample.

## III. RESULTS AND DISCUSSIONS

### A. Toxicity and $LD_{50}$ Values

The general toxicity together with the CCNSC (Cancer Chemotherapy National Service Center) toxicity reports are listed in Table 1 and the $LD_{50}$ values determined for the selected plant species are listed in Table 2.

Toxicity of aquatic plants in general is relatively low. The $LD_{50}$ for the most toxic one, i.e., N. tuberosa (stem), in mice was 3 gm of dry plant meterial/kg (ca. 25.4 gm of wet plant material/kg). Signs exhibited by leaves and stems extracts of N. tuberosa were quite different; crouching and respiratory exhaustion for the leaf extract, whereas tonic convulsion and increased respiratory rate were observed for the stem extract. Distinct toxic principles might be present in leaf and stem or one toxic constituent which exerts different actions depending on the doses administered was responsible for the two different signs observed. Odintsova reported (8) N. alba toxicity in dogs was quite low (dogs can tolerate 100 ml of 6% extract three times a day for 45 days) and that the 1:100,000 infusion raised the blood pressure in rabbits.

### B. Antineoplastic

The antineoplastic properties of various extracts against amelanoma tumor and the CCNSC screening test reports are shown in Tables 3 and 4 respectively.

Table 1. Toxicity in Aquatic Plants.

| Plant[1/] | Mortality[2/] | CCNSC[3/] |
|---|---|---|
| Ac-1 | 1/6 | 3/18 |
| Ac-2 | 1/6 | |
| Cd | 4/6 | 7 18 |
| Cl | 3/6 | 1/18 |
| Cp | 2/6 | |
| Cv | 2/6 | 4/18 |
| Es | 1/6 | 0/18 |
| Lm | 3/6 | 2/18 |
| Me | 3/6 | 7/18 |
| Nt lv | 2/6 | 11/18 |
| Nt st | 4/6 | 13/18 |
| Nv lv-1 | 2/6 | |
| Nv st-1 | 1/6 | 0/18 |
| Nv lv-2 | 1/6 | |
| Nv st-2 | 1/6 | |
| Pa | 1/6 | 9/18 |
| Pn | 3/6 | 0/18 |
| Pp | 2/6 | 8/18 |
| Pr | 1/6 | 1/18 |
| Pz | 2/6 | |
| Sc | 1/6 | |
| Se | 1/6 | |
| Sf | 1/6 | 4/18 |
| Sl | 3/6 | |
| Ta-1 | 0.6 | |
| Ta-2 | 1/6 | |
| Va | 2/6 | 2/18 |
| Za | 1/6 | 5/18 |

1/Plant names: (refer to section "II. Materials and Methods").
2/Mortality was expressed as the accumulated numbers of death of Swiss Webster mice/total number of experimental animals. The dose administered was 3.0 gm of dry plant material/kg.
3/CCNSC toxic report on $CDF_1$ and $BDF_1$ mice. The dose level was 400 mg of 50% ethanol extract/kg.



Table 2. Toxic Reactions and the $LD_{50}$ Values of C. demersum and N. tuberosa (leaf and stem).

| Plant | Dose[1/] (gm/kg) | Mortality ratio | Probit | $LD_{50}$ (gm/kg)[1/] | Type of action |
|---|---|---|---|---|---|
| Cd | 7.50 | 9/10 | 6.28 | 4.25±0.68 | stretching and increased respiratory rate |
| | 3.75 | 5/10 | 5.00 | | |
| | 1.87 | 0/10 | 3,04 | | |
| Nt lv | 12.00 | 8/10 | 5.84 | 6±1.88 | crouching and respiratory exhaustion |
| | 6.00 | 5/10 | 5.00 | | |
| | 3.00 | 2/10 | 4.16 | | |
| Nt st | 5.40 | 8/10 | 5.85 | 3±1.13 | tonic convulsion and increased respiratory rate |
| | 2.70 | 5/10 | 5.00 | | |
| | 1.35 | 2/10 | 4.16 | | |

1/Dose and $LD_{50}$ were expressed as gm of dry plant material/kg.

Table 3. Antineoplastic Activity Against Amelanoma Cells.

| Plant[1/] | Ext.[2/] | Dose (gm/kg)[3/] | Lag period (days) | % Inhibition[4/] |
|---|---|---|---|---|
| Ac-1 | S+C | 6.25 | 8 | |
| | A | 3.75 | 8 | 14 |
| | W | 1.88 | 7 | |
| Ac-2 | S+C | 5.80 | 8 | |
| | A | 3.75 | 7 | |
| | W | 1.88 | 7 | |
| Cd | S+C | 3.75 | 8 | |
| | A | 1.88 | 8 | |
| | W | 0.94 | 8 | |
| Cl | S+C | 15.00 | 9 | |
| | A | 3.75 | 8 | 10 |
| | W | 3.75 | 7 | |
| Cp | S+C | 6.20 | 8 | 18 |
| | A | 3.75 | 7 | 15 |
| | W | 1.88 | 7 | |
| Cv | S+C | 18.80 | 9 | |
| | A | 3.75 | 7 | 15 |
| | W | 1.88 | 7 | 10 |
| Es | S+C | 6.25 | 6 | 12 |
| | A | 3.75 | 7 | 17 |
| | W | 3.75 | 7 | |
| Lm | S+C | 4.00 | 9 | 22 |
| | A | 3.75 | 7 | 2 |
| | W | 1.88 | 6 | |
| Me | S+C | 25.00 | 8 | |
| | A | 3.75 | 8 | 14 |
| | W | 1.88 | 6 | |
| Nt | S+C | 2.70 | 8 | |
| lv | A | 3.75 | 6 | 8 |
| | W | 1.88 | 7 | 14 |
| Nt | S+C | 3.75 | 8 | 20 |
| St | A | 3.75 | 8 | 13 |
| | W | 1.88 | 9 | 14 |
| Nv-1 | S+C | 3.60 | 9 | |
| lv | A | 1.88 | 7 | |
| | W | 1.88 | 7 | |
| Nv-1 | S+C | 5.80 | 8 | 43 |
| st | A | 3.75 | 7 | 38 |
| | W | 3.75 | 6 | |
| Nv-2 | S+C | 6.30 | 10 | 26 |
| lv | A | 3.75 | 9 | 18 |
| | W | 3.75 | 9 | 53 |
| Nv-2 | S+C | 10.00 | 10 | 54 |
| st | A | 3.75 | 8 | 14 |
| | W | 3.75 | 8 | 6 |
| Pa | S+C | 7.50 | 8 | |
| | A | 3.75 | 8 | |
| | W | 1.88 | 7 | |



(Table 3 continued)

| Plant[1/] | Ext.[2/] | Dose (gm/kg)[3/] | Lag period (days) | % Inhibition[4/] |
|---|---|---|---|---|
| Pn | S+C | 1.60 | 8 | 8 |
| | A | 3.75 | 8 | |
| | W | 1.88 | 7 | 8 |
| Pp | S+C | 10.00 | 8 | 18 |
| | A | 3.75 | 7 | 15 |
| | W | 1.88 | 6 | |
| Pr | S+C | 5.00 | 7 | 10 |
| | A | 3.75 | 7 | 12 |
| | W | 1.88 | 8 | |
| Pz | S+C | 25.00 | 8 | |
| | A | 3.75 | 6 | 27 |
| | W | 1.88 | 8 | |
| Sc | S+C | 4.20 | 7 | 28 |
| | A | 3.75 | 7 | |
| | W | 1.88 | 8 | 15 |
| Se | S+C | 12.50 | 7 | 43 |
| | A | 3.75 | 8 | |
| | W | 1.88 | 8 | 7 |
| Sf | S+C | 10.00 | 7 | 43 |
| | A | 3.75 | 7 | |
| | W | 1.88 | 7 | |
| Sl | S+C | 3.00 | 7 | |
| | A | 3.75 | 6 | |
| | W | 1.88 | 7 | 7 |
| Ta-1 | S+C | 1.20 | 7 | 31 |
| | A | 3.75 | 7 | 17 |
| | W | 3.75 | 6 | |
| Ta-2 | S+C | 5.00 | 6 | |
| | A | 3.75 | 8 | |
| | W | 1.88 | 8 | 42 |
| Va | S+C | 3.80 | 8 | 33 |
| | A | 3.75 | 7 | |
| | W | 1.88 | 7 | 39 |
| Za | S+C | 10.00 | 6 | |
| | A | 3.75 | 7 | |
| | W | 0.94 | 7 (average) | |
| Control | | | | |

1/Plant names: (refer to section "II. Materials and Methods").
2/Extracts: S+C- skellysolve F and chloroform combined; A- 80% ethanol; W- acidic and basic water combined.
3/Dose was expressed as the grams of dried plant material vs. kg body weight of hamsters.
4/Percent inhibition was obtained as follows: average tumor wt. (control) minus tumor wt. (treated)/average tumor wt. (control). Underlined % inhibitions were for repeating results on four more hamsters.

Table 4. Summary of the CCNSC Screening Test Reports[1/].

| I | II[2/] | III[3/] | IV[4/] | V[5/] | VI[6/] | VII[7/] | VIII[8/] | IX[9/] |
|---|---|---|---|---|---|---|---|---|
| Ac | 06 | LE | 2 | 2 | 1 | 400 | 101 | 02 |
| | 02 | LE | 2 | 9 | 1 | 400 | 101 | 21F |
| | 02 | PS | 3 | 2 | 1 | 400 | 100 | 02 |
| | 90 | KB | 5 | B | | -0.0 | $1.0x10^2$ | 02 |
| Cd | 06 | LE | 2 | 2 | 1 | 400 | | 01 |
| | 02 | LE | 2 | 2 | 1 | 200 | 95 | 04 |
| | 02 | LE | 2 | 9 | 1 | 400 | 98 | 21F |
| | 02 | PS | 3 | 2 | 1 | 400 | 100 | 02 |
| | 90 | KB | 5 | B | | -0.0 | $1.0x10^2$ | 02 |
| Cl | 06 | LE | 2 | 2 | 1 | 400 | 103 | 02 |
| | 02 | LE | 2 | 2 | 1 | 400 | 96 | 21F |
| | 06 | PS | 3 | 2 | 1 | 400 | 100 | 02 |
| | 90 | KB | 5 | B | | 00.43 | $4.4x10^2$ | 02 |
| Cv | 06 | LE | 2 | 2 | 1 | 400 | 100 | 02 |
| | 02 | LE | 2 | 2 | 1 | 400 | 98 | 21F |
| | 06 | PS | 3 | 2 | 1 | 400 | | 01 |
| | 02 | PS | 3 | 2 | 1 | 200 | 100 | 04 |
| | 90 | KB | 5 | B | | -0.43 | $1.2x10^2$ | 02 |
| Es | 06 | LE | 2 | 2 | 1 | 400 | 102 | 02 |
| | 02 | LE | 2 | 9 | 1 | 400 | 103 | 21F |
| | 02 | PS | 3 | 2 | 1 | 400 | 86 | 02 |
| | 06 | PS | 3 | 2 | 1 | 200 | 100 | 02E |
| | 90 | KB | 5 | B | | -0.0 | $1.0x10^2$ | 02 |
| Lm | 06 | LE | 2 | 2 | 1 | 400 | 93 | 02 |
| | 02 | LE | 2 | 9 | 1 | 400 | 98 | 21F |
| | 02 | PS | 3 | 2 | 1 | 400 | | 01 |
| | 06 | PS | 3 | 2 | 1 | 200 | 100 | 04 |
| | 90 | KB | 5 | B | | -0.0 | $1.0x10^2$ | 02 |
| Me | 06 | LE | 2 | 2 | 1 | 400 | 93 | 02 |
| | 02 | LE | 2 | 9 | 1 | 400 | 98 | 21F |
| | 02 | PS | 3 | 2 | 1 | 400 | | 01 |
| | 06 | PS | 3 | 2 | 1 | 100 | 100 | 04 |
| | 90 | KB | 5 | B | | -0.0 | $1.0x10^2$ | 02 |
| Nt | 06 | LE | 2 | 2 | 1 | 400 | | 01 |
| lv | 06 | LE | 2 | 2 | 1 | 100 | 100 | 04 |
| | 02 | LE | 2 | 9 | 1 | 400 | 97 | 21F |
| | 02 | PS | 3 | 2 | 1 | 400 | | 01 |
| | 06 | PS | 3 | 2 | 1 | 100 | 100 | 04 |
| | 90 | KB | 5 | B | | -0.0 | $1.0x10^2$ | 02 |
| Nt | 06 | LE | 2 | 2 | 1 | 150 | | 01 |
| st | 06 | LE | 2 | 2 | 1 | 37.5 | 97 | 04 |
| | 02 | LE | 2 | 9 | 1 | 400 | | 21 |
| | 06 | LE | 2 | 2 | 1 | 300 | | 21F |
| | 06 | LE | 2 | 2 | 1 | 150 | 113 | 27F |
| | 06 | LE | 2 | 2 | 1 | 75.0 | 106 | 27F |
| | 06 | PS | 3 | 2 | 1 | 150 | | 01 |
| | 06 | PS | 3 | 2 | 1 | 37.5 | 100 | 04 |
| | 90 | KB | 5 | B | | -0.0 | $1.0x10^2$ | 02 |



(Table 4 Continued)

| I | II | III | IV | V | VI | VII | VIII | IX |
|---|---|---|---|---|---|---|---|---|
| Nv | 06 | LE | 2 | 2 | 1 | 400 | 101 | 02 |
| st | 02 | LE | 2 | 9 | 1 | 400 | 95 | 21F |
| | 02 | PS | 3 | 2 | 1 | 400 | | 01 |
| | 06 | PS | 3 | 2 | 1 | 200 | 100 | 04 |
| | 80 | KB | 5 | B | | -0.0 | $1.0x10^2$ | 02 |
| Pa | 06 | LE | 2 | 2 | 1 | 400 | | 01 |
| | 06 | LE | 2 | 2 | 1 | 200 | 98 | 04 |
| | 02 | LE | 2 | 9 | 1 | 400 | 98 | 21F |
| | 02 | PS | 3 | 2 | 1 | 400 | | 01 |
| | 06 | PS | 3 | 2 | 1 | 100 | | 03 |
| | 02 | PS | 3 | 2 | 1 | 50.0 | 87 | 04 |
| | 90 | KB | 5 | B | | -1.06 | $2.7x10^2$ | 02 |
| Pn | 06 | LE | 2 | 2 | 1 | 400 | 101 | 02 |
| | 02 | LE | 2 | 9 | 1 | 400 | 97 | 21F |
| | 02 | PS | 3 | 2 | 1 | 400 | 90 | 02 |
| | 90 | KB | 5 | B | | -0.0 | $1.0x10^2$ | 02 |
| Pp | 06 | LE | 2 | 2 | 1 | 400 | | 01 |
| | 06 | LE | 2 | 2 | 1 | 100 | 102 | 04 |
| | 02 | LE | 2 | 9 | 1 | 400 | 101 | 21F |
| | 02 | PS | 3 | 2 | 1 | 400 | | 01 |
| | 06 | PS | 3 | 2 | 1 | 200 | 100 | 04 |
| | 90 | KB | 5 | B | | -0.0 | $1.0x10^2$ | 02 |
| Pr | 06 | LE | 2 | 2 | 1 | 400 | 96 | 02 |
| | 02 | LE | 2 | 9 | 1 | 400 | 101 | 21F |
| | 02 | PS | 3 | 2 | 1 | 400 | | 01 |
| | 06 | PS | 3 | 2 | 1 | 200 | 100 | 04 |
| | 90 | KB | 5 | B | | -0.0 | $1.0x.0^2$ | 02 |
| Sf | 06 | LE | 2 | 2 | 1 | 400 | 98 | 02 |
| | 02 | LE | 2 | 9 | 1 | 400 | 101 | 21F |
| | 02 | PS | 3 | 2 | 1 | 400 | | 01 |
| | 06 | PS | 3 | 2 | 1 | 200 | 100 | 04 |
| | 90 | KB | 5 | B | | -0.0 | $1.0x10^2$ | 02 |
| Va | 06 | LE | 2 | 2 | 1 | 400 | 96 | 02 |
| | 02 | LE | 2 | 9 | 1 | 400 | 101 | 21F |
| | 02 | PS | 3 | 2 | 1 | 400 | 95 | 02 |
| | 90 | KB | 5 | B | | -0.0 | $1.0x10^2$ | 02 |
| Za | 06 | LE | 2 | 2 | 1 | 400 | | 01 |
| | 06 | LE | 2 | 2 | 1 | 200 | 102 | 04 |
| | 02 | LE | 2 | 9 | 1 | 400 | 101 | 21F |
| | 02 | PS | 3 | 2 | 1 | 400 | | 01 |
| | 06 | PS | 3 | 2 | 1 | 200 | 100 | 04 |
| | 90 | KB | 5 | B | | -0.0 | $1.0x10^2$ | 02 |

1/I- Plant names (refer to section "II. Materials and Methods"); II- Host; III- Tumor; IV- Parameter; V- Vehicle; VI- Route; VII- Dose; VIII- Evaluation; IX- Test status code.
2/Host 02- $BDF_1$ mice; 06- $CDF_1$ mice; 90- Cell culture tube assay.
3/Tumor KB- Human epidermoid carcinoma of the nasopharynx; LE- L-1210 lymphoid leukemia; PS- P388 lymphocytic leukemia.
4/Parameter 2- Mean survival time; 3- Median survival time; 5- $ED_{50}$ (concentration causing 50% inhibition of protein synthesis.
5/Vehicle 2- Saline; 9- Water; B- Propylene glycol.
6/Route 1- Intraperitoneal.
7/Dose- In mg/kg, except for KB cell culture tube assay where change of response for each one-log change of dose was expressed as W (mg/ml) slope -0.0, and so on.
8/Evaluation- Percent (T/C) % (i.e., ratio of survival time of test animals to control animals, expressed as %) for No. 2 and 3 parameter; $ED_{50}$ (mg/ml) for No. 5 parameter.
9/Test status code 01- Toxic effect; 02- Non-toxic inactive; 02E- Non-toxic inactive, exception to routine testing procedure; 03- Toxic effect; 04- Non-toxic inactive; 21F- Single test assay, activity failed criteria; 27F- Multiple dose assay-single treatment, activity failed criteria.

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Extracts which failed to give greater than 50% tumor-inhibition activity and prolongation of more than two-day lag period were considered as negative. According to this criterium, only Nuphar variegatum indicated an anticancer possibility, the remaining aquatic plants have no significant inhibition activity at the doses indicated on amelanoma cells. CCNSC screening results of 50% ethanol extracts on KB-human epidermoid carcinoma of the nasopharynx, LE-L-1210 lymphoid leukemia and PS-P388 lymphocytic leukemia were not encouraging. Although the results were either non-toxic or the antineoplastic activity failed the CCNSC criteria for further investigation, it is important to include these negative data for the benefit of other investigators.

C. Anticoagulation

The anticoagulation effects in terms of prothrombin and partial thromboplastin time are shown in Table 5.

Normal prothrombin time (PT) for mice injected with 0.9% NaCl was 18 seconds. For those PTs which were longer than 23 seconds and shorter than 13 seconds were considered being prolonged or shortened. Prolongation of PT was observed in 80% ethanol extracts of Carex lacustris, Eleocharis smillii, Myriophyllum exalbescens, Nuphar variegatum, Nymphaea tuberosa, Potamogeton natans, P. richardsonii, P. zosteriformis, Sagittaria latifolia and Zizania aquatica, whereas a slight shortening of PT was observed in the skellysolve F and chloroform extracts of Carex lacustris (12 sec.) and Potamogeton pectinatus (12 sec.). Normal partial thromboplastin time (PTT) for mice was 51 seconds and only the prolongation of PTT (longer than 61 seconds) was observed in 50% of the aquatic plants tested. Among these plants, the most significant increase of PTT (more than 20 minutes) was observed in Carex lacustris, Myriophyllum exalbescens, Nuphar variegatum and Nymphaea tuberosa.



Table 5. Effect of Various Extracts on Prothrombin Time (PT) and Partial Thromboplastin Time (PTT).

| Plant[1/] | Ext.[2/] | PT (min-sec)[3/] | PTT (min-sec)[3/] | Plant | Ext. | PT (min-sec) | PTT (min-sec) |
|---|---|---|---|---|---|---|---|
| Ac-1 | S+C | 0-17 | 0-52 | Nv-2 | S+C | 0-13 | 1-04 |
| | A | 0-22 | 0-43 | st | A | 0-19 | 1-56 |
| Ac-2 | S+C | 0-13 | 1-02 | Pa | S+C | 0-16 | 1-45 |
| | A | 0-22 | 0-56 | | A | 0-17 | 2-56 |
| Cd | S+C | 0-19 | 1-04 | Pn | S+C | 0-17 | 0-55 |
| | A | 0-16 | 0-41 | | A | 0-27 | 0-47 |
| Cl | S+C | 0-12 | 1-42 | Pp | S+C | 0-12 | 0-53 |
| | A | 2-01 | MT20-00 | | A | 0-14 | 0-57 |
| Cp | S+C | 0-14 | 1-01 | Pr | S+C | 0-13 | 0-48 |
| | A | 0-18 | 4-34 | | A | 0-48 | 0-42 |
| Cv | S+C | 0-15 | 0-51 | Pz | S+C | 0-18 | 1-41 |
| | A | 0-17 | 0-48 | | A | 0-34 | 5-29 |
| Es | S+C | 0-14 | 0-53 | Sc | S+C | 0-19 | 1-28 |
| | A | 0-47 | 0-58 | | A | 0-19 | 0-53 |
| Lm | S+C | 0-15 | 1-18 | Se | S+C | 0-15 | 0-57 |
| | A | 0-20 | 1-15 | | A | 0-15 | 0-48 |
| Me | S+C | 0-15 | 2-38 | Sf | S+C | 0-18 | 0-53 |
| | A | 2-15 | MT20-00 | | A | 0-16 | 0-56 |
| Nt | S+C | 0-14 | 1-08 | Sl | S+C | 0-15 | 2-29 |
| lv | A | MT20-00 | MT20-00 | | A | 4-42 | 0-52 |
| Nt | S+C | 0-16 | 1-27 | Ta-1 | S+C | 0-14 | 0-55 |
| st | A | MT20-00 | MT20-00 | | A | 0-20 | 0-55 |
| Nv-1 | S+C | 0-22 | 1-33 | Ta-2 | S+C | 0-17 | 0-54 |
| lv | A | MT20-00 | MT20-00 | | A | 0-18 | 0-42 |
| Nv-1 | S+C | 0-15 | 1-10 | Va | S+C | 0-17 | 0-54 |
| st | A | MT20-00 | MT20-00 | | A | 0-18 | 0-49 |
| Nv-2 | S+C | 0-14 | 2-23 | Za | S+C | 0-16 | 1-11 |
| lv | A | 0-21 | 1-45 | | A | 0-27 | 10-56 |

1/Plant names- (refer to section "II. Materials and Methods").
2/Extracts S+C- skellysolve F and chloroform combined; A- 80% ethanol.
3/For those extracts which failed to have any clot formation after 20 minutes are recorded as MT20 (more than 20 minutes).

The anti-coagulants present in the extracts could have affected various factors involved in the intrinsic and extrinsic blood coagulation systems. The technique used does not enable us to pin point the exact factors affected. The shortening or prolongation of PT or PTT may also be observed in mice which have liver diseases or congenital factor deficiencies.

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