PHYTOCHEMICAL ANALYSIS AND COAGULATION EFFECT OF *C.AURANTIFOLIA* SWINGLE LEAVES AND *A.FISTULOSUM*

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ABSTRACT

The present study uncovered the potential pharmacological activities of ethanol extracts from Allium fistulosum L (*A. fistulosum*) and *Citrus aurantifolia* (*C. aurantifolia*) leaves. Phytochemical analysis revealed that *C. aurantifolia* contains various phytoconstituents, including polyphenols and flavonoids.

In this research, the impact of administering ethanol extracts of *Citrus aurantifolia* and *A. fistulosum* was assessed on several coagulation parameters, including Prothrombin time (PT), activated partial thromboplastin time (aPTT), and thrombin time (TT) in rabbits. These assessments were conducted at three different doses: 200 mg/kg, 400 mg/kg, and 600 mg/kg body weight, following 30 and 45 days of administration. As a benchmark, warfarin was administered at a standard dose of 0.54 mg/kg body weight.

Animals that received *A. fistulosum* at a dose of 400 mg/kg body weight displayed a significant increase in TT, PT, and aPTT. In contrast, animals administered *A. fistulosum* showed a noteworthy increase in PT and a highly significant increase in TT and aPTT.

Animals that received a dosage of 400 mg/kg body weight of *Citrus aurantifolia* swingle leaves extract exhibited a noteworthy increase in TT, PT, and aPTT. Meanwhile, animals administered a dosage of 600 mg/kg body weight of *Citrus aurantifolia* swingle leaves extract displayed a noteworthy increase in TT, PT, and a highly significant increase in aPTT.

Furthermore, animals that were given warfarin at a dosage of 0.54 mg/kg body weight exhibited a highly significant increase in all parameters (TT, PT, aPTT, and Fg).

Keywords: Phytochemical, flavonoid, anti-coagulant, therapeutic

INTRODUCTION

Citrus limon, commonly known as lemon, is believed to have its origins in Asia, specifically in regions like China, Myanmar, and Northeast India, particularly Assam. Taxonomically, it belongs to the Rutaceae family. Lemon cultivation thrives in tropical and subtropical areas characterized by high humidity and winter precipitation, making regions like southern Brazil and Florida ideal for commercial citrus production. Unpruned lemon trees can grow to heights of up to 6 meters (approximately 10 to 20 feet). The leaves of lemon trees initially appear reddish and turn green as they mature. It's important to note that various lemon cultivars can display distinct characteristics, including the presence of thorns or spines on new shoots emerging from the leaf axils. This

information can be relevant in a medical article, as lemon is known for its potential health benefits and medicinal uses (1).

In lemon trees, the flowers typically appear at the junction of the leaves, featuring petals that are primarily white in the lower portion and reddish in the upper section (2). The lemon fruit is distinctive with eight to ten lobes and a rounded, open tip. The fruit is elongated and segmented, with oil glands visible beneath its thick peel (3).

The Rutaceae family, the largest within the order Sapindales, includes over 2100 species across 154 genera, with Citrus being economically important. Thriving in tropical and subtropical climates, it shows diverse species in South and Central America, Southern America, New Zealand, New Caledonia, and nearby Pacific archipelagos. Predominantly comprised of large shrubs or trees, some smaller genera feature herbaceous species (2).

With few exceptions, most Rutaceae species, like Phellodendron Rupr, have cavities. Notably, the Rutaceae family displays significant morphological diversity, including variations in fruit types, such as detached or baccate fruits, indicating elastic seed dispersion mechanisms, as observed in the Citrinae and Aurantioideae Eaton subfamilies (2, 4).

Apart from fruits, the Rutaceae family yields valuable products such as samaras and dupes. While Engler's 1896 classification originally relied on fruit characteristics to categorize Rutaceae into six or seven divisions, contemporary morphological and phylogenetic investigations have demonstrated that fruit traits alone do not provide adequate differentiation for the Rutaceae family (5, 6)

Therefore, considering their unpredictable nature, other physical attributes become more important in classifying species within the family (7).

MATERIAL AND METHODS

Phytochemical Analysis of *Citrus aurantifolia* Swingle Leaves

i. **Test for Alkaloids (Wagner Test):** Alkaloids were detected by observing the formation of a reddish-brown precipitate when mixing crude extracts with Wagner's reagent.

ii. **Triterpenoids and Steroids:** A solution containing 20 cc of ethanol and test extracts was heated in a water bath. After filtration, vaporization, and the addition of 10 ml of diethyl ether, another round of filtration was performed. The resulting filtrate was allowed to naturally dry at room temperature. The presence of triterpenoids was indicated by the appearance of a red color upon the addition of a small quantity of concentrated sulfuric acid and three to five drops of acetic acid. Conversely, the presence of steroids was indicated by a blue or greenish-blue color.

iii. **Saponins (Foam Test):** Crude extracts were boiled while mixed with distilled water, and the formation of bubbles indicated the presence of saponins**.**

iv. **Flavonoids (H2SO4 Test):** By combining the crude extracts with a small amount of H2SO4, the development of an orange hue served as an indicator of flavonoids.

v. **Phlobatannins (5 phalobatannins):** Filtered crude extracts were diluted in distilled water and heated in 2 ml of HCl solution. The formation of a crimson precipitate indicated the presence of phlobatannins.

vi. **Sugar Reduction Test:** Crude extracts, after being mixed with distilled water and shaken, were filtered. The filtrate was then boiled for a few minutes while adding Fehling's solution in small amounts. The formation of an orange-red precipitate indicated the presence of reducing sugar.

vii. **Carbohydrate Test (Iodine Test):** Prior to filtration, 5 ml of distilled water was used to dilute the raw extracts. Subsequently, the filtrate was boiled in a water bath while a few drops of FeCl3 were added.

viii. **Phenolic Test:** To dissolve the crude extracts, a mixture of 5 cc of distilled water and a small amount of 10% iron (III) chloride was employed. The introduction of this solution to the mixture resulted in a color change to green or blue, indicating the presence of phenolic content.

ix. **Test for Glycosides:** After diluting the crude extracts in 20 ml of distilled water, a few drops of bromine were added. The formation of a yellow precipitation served as an indicator of the presence of glycosides.

Parameters for Coagulants

i. **Thromboplastin Time:**

The Hemostat Thromboplastin-SI was used for both manual and automated determination of prothrombin time.

The principle involved the presence of tissue factor and recalcified plasma, leading to the activation of factor Xa and thrombin, resulting in fibrinolysis and the formation of an insoluble fibrin clot. To dissolve the crude extracts, they were mixed with 20 ml of distilled water, followed

by the addition of 3-5 drops of bromine. The presence of glycosides was indicated by the formation of a yellow precipitate.

The reagent used consisted of lyophilized thromboplastin reagent containing components like 2.6% Brain extract of Rabbit, 0.13% Calcium Chloride, salt, and stabilizers. This reagent typically initiates the clotting process by activating the intrinsic pathway of coagulation.

To prepare the reagent, 2.0 ml of distilled water was added to the vial, gently stirred, and left at room temperature for 15 minutes. Then, the reconstituted reagent was transferred into a pre-warmed test tube.

Plasma was used in this step, with an incubation period of 3-5 minutes at 37°C.

A pre-warmed reagent was introduced, and the timer was initiated upon addition to record the clot formation time.

ii. Activated Partial Thromboplastin Time:

The Hemostat aPTT-EL, using elagic acid as a triggering agent, was employed to evaluate the activated partial thromboplastin time (aPTT) either manually or mechanically. This test assessed coagulation factors within the common and intrinsic pathways.

The principle involved mixing the aPTT-EL reagent with a plasma activator and phospholipids acting as a platelet replacement. After an activation incubation, calcium chloride was added to initiate clot formation.

Reagent 1 contained 0.007% Phospholipids from rabbit brain and 0.0037% Ellagic acid, along with additional ingredients like buffers, salts, and stabilizers. Reagent 2 was a solution of 78 0.02 mol/l CaCl2 with additional salts and stabilizers.

To prepare the reagents, they were transferred into pre-warmed test tubes.

Plasma was utilized in the procedure, and an incubation period of 1-2 minutes at 37°C was observed.

Reagent 1 was introduced, followed by another incubation for 3-5 minutes at 37°C.

Then, 0.1 ml of pre-warmed reagent 2 was added.

The timer was initiated after adding the reagent, and the time taken for clot formation was recorded.

iii. Thrombin Time:

Hemostat Thrombin Thrombin measured thrombin time both manually and automatically.

The principle involved the assessment of variables that could affect the conversion of fibrinogen to fibrin. In this process, undiluted plasma was mixed with low-potency thrombin, leading to the formation of a clot.

The reagent contained buffers, lyophilized thrombin reagent, and bovine thrombin at approximately 10 NIH units per milliliter.

To prepare the reagents, the lyophilized thrombin reagent was reconstituted with 1.0 ml of distilled water, gently stirred, and transferred to a pre-warmed test tube.

Plasma was used in the procedure, with an incubation period of 3 minutes at 37 ºC.

Then, 0.1 ml of the reagent was added to the mixture.

The timer was started immediately upon adding the reagent, and the time of clot formation was recorded.

iv. Fibrinogen Test:

The Hemostat Fibrinogen test allowed for the determination of fibrinogen concentration in a patient's blood, conducted either manually or through an automated procedure.

The principle involved a diluted sample of plasma mixed with bovine thrombin. The time taken for the sample to clot was inversely related to the fibrinogen content.

Reagent 1 included thrombin reagent, lyophilized bovine thrombin, imidazole buffered salts, and stabilizers.

To prepare the reagents, the lyophilized thrombin reagent was reconstituted with 2 ml of distilled water, and the buffer was prepared.

A 1:10 dilution of plasma was made using the buffer by adding 100 l of plasma to 900 l of buffered saline.

The procedure included transferring 0.2 ml of the diluted sample into a pre-warmed test tube, incubating for 4-6 minutes at 37℃, adding 0.1 ml of reagent, starting the timer upon adding the reagent, and recording the time when clot formation became evident.

RESULTS

Preliminary Phytochemical Screening

Phytochemical analysis of the ethanol extract from *C. aurantifolia* swingle leaves was performed to identify its chemical components. The results indicated the presence of alkaloids, triterpenoids, steroids, saponins, flavonoids, reducing sugars, carbohydrates, phenolic compounds, and tannins. A positive result indicated the presence of a specific phytoconstituent, while a negative result indicated its absence.

TABLE 1: Phytochemical screening of *C.aurantifolia leaves*

Phytochemical test	**Result**
Alkaloids	Positive
Triterpenoids	**Negative**
Steroids	**Positive**
Flavonoids	**Positive**
Phlobatannins	**Negative**
Reducing Sugar	**Positive**
Carbohydrates	**Positive**
Phenolic Content	**Positive**
Glycosides	**Positive**
Tannins	**Positive**

Coagulation Parameters

Table 2 presents the effects of continuous administration of ethanol extracts from A.fistulosum and C.aurantifolia swingle leaves for 30 days on PT, aPTT, TT, and Fg.

Animals receiving A.fistulosum ethanol extract at a dose of 200mg/kg body weight showed minimal changes in all parameters (TT, PT, aPTT, and Fg) compared to the control group. The recorded values were TT - 10.85±0.42, PT - 12.23±2.7, aPTT - 10.31±0.67, and Fg - 28.34±0.37, while the control group values were TT - 9.32±0.35, PT - 11.66±1.6, aPTT - 9.10±0.34, and Fg - 27.5±1.6.

In contrast, animals treated with A.fistulosum ethanol extract at a dose of 400mg/kg body weight displayed a significant increase in TT and aPTT (12.35±0.42 and 13.41±0.21, respectively) compared to the control group. However, the changes in PT and Fg were not statistically significant (13.64±0.43 and 30.67±0.14, respectively) compared to the control group.

Animals administered with A.fistulosum ethanol extract at a dose of 600mg/kg body weight demonstrated a notable increase in all parameters (TT, PT, aPTT, and Fg) compared to the control group. The recorded values were TT - 13.85±0.82, PT - 16.43±0.32, aPTT - 16.33±0.46, and Fg -

34.56±2.22, while the control group values were TT - 9.32±0.35, PT - 11.66±1.6, aPTT - 9.10±0.34, and Fg - 27.5±1.6.

Similarly, animals administered with C.aurantifolia swingle leaves extract at a dose of 200mg/kg body weight displayed minimal changes in all parameters (TT, PT, aPTT, and Fg) compared to the control group. The recorded values were TT - 11.45±0.34, PT - 13.43±1.3, aPTT - 10.91±2.1, and Fg - 29.54±0.32, while the control group values were TT - 9.32±0.35, PT - 11.66±1.6, aPTT - 9.10±0.34, and Fg - 27.5±1.6.

Animals treated with C.aurantifolia swingle leaves extract at a dose of 400mg/kg body weight displayed a significant increase in TT and aPTT (13.56±0.21 and 14.43±0.54, respectively) compared to the control group. However, the changes in PT and Fg were not statistically significant (13.67±0.78 and 30.21±0.34, respectively) compared to the control group.

Animals administered with C.aurantifolia swingle leaves extract at a dose of 600mg/kg body weight also demonstrated a notable increase in all parameters (TT, PT, aPTT, and Fg) compared to the control group. The recorded values were TT - 15.40±0.77, PT - 16.78±0.45, aPTT - 16.88±0.32, and Fg - 33.21±0.21, while the control group values were TT - 9.32±0.35, PT - 11.66±1.6, aPTT - 9.10±0.34, and Fg - 27.5±1.6.

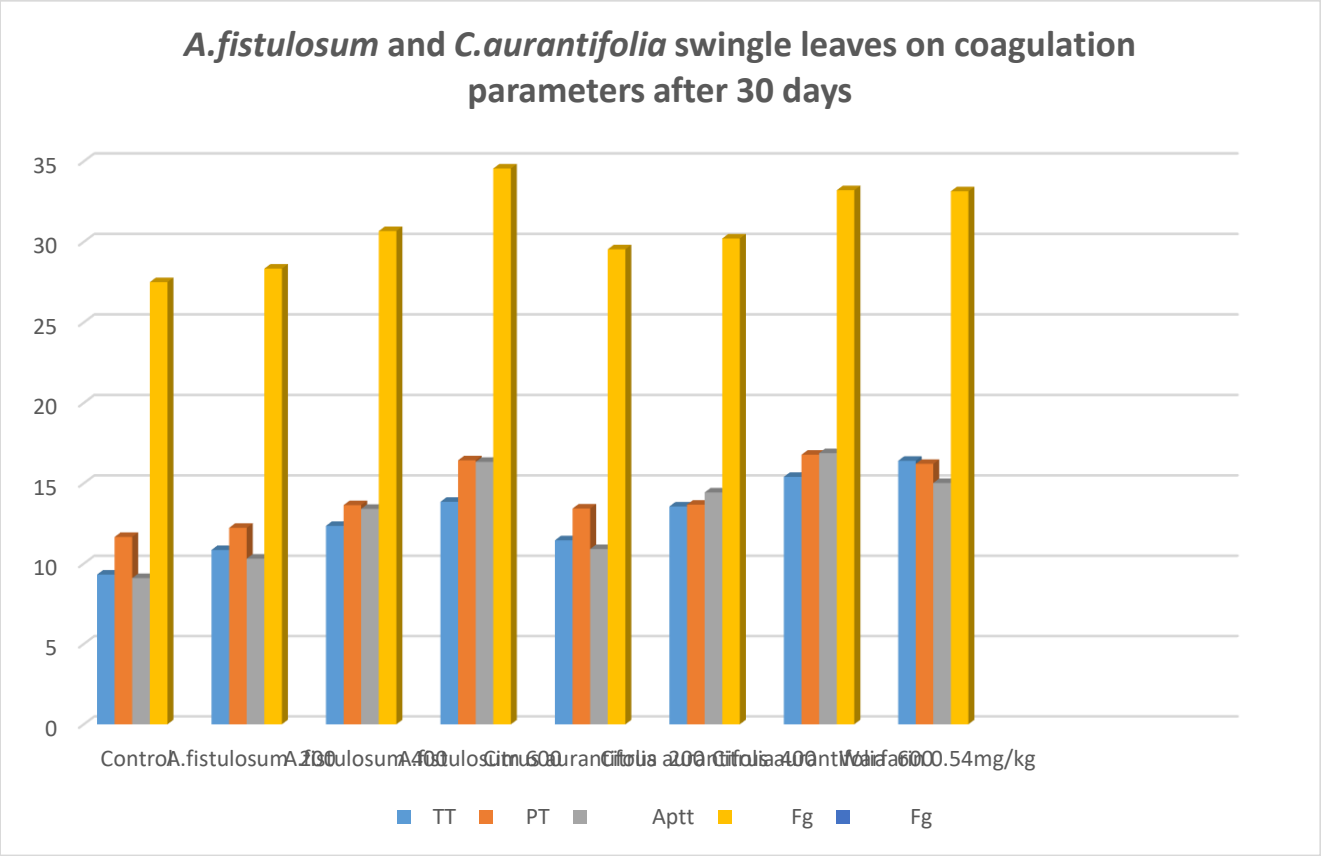
Additionally, animals administered with the standard drug warfarin at a dose of 0.54mg/kg body weight also exhibited a significant increase in all parameters (TT, PT, aPTT, and Fg) compared to the control group. The recorded values were TT - 16.40±1.1, PT - 16.21±1.1, aPTT - 15.02±0.69, and Fg - 33.14±1.5, while the control group values were TT - 9.32±0.35, PT - 11.66±1.6, aPTT - 9.10±0.34, and Fg - 27.5±1

Table 2: Impact of *A. fistulosum* and *C. aurantifolia* Swingle leaves on coagulation parameters after a 45-day duration.

Parameters	TT	PT	aPTT	Fg
Control	9.32 ± 0.34	11.67 ± 1.6	9.11 ± 0.34	27.6 ± 1.6
***A.fistulosum* 200**	10.85±0.41	12.24±2.7	10.32±0.67	28.35±0.37
***A.fistulosum* 400**	12.35±0.4*	13.65±0.43	13.42±0.21*	30.68±0.14
***A.fistulosum* 600**	13.85±0.85*	16.42±0.32*	16.34±0.46*	34.55±2.22*
Citrus aurantifolia 200	11.45 ± 0.32	13.42 ± 1.3	10.92 ± 2.1	29.53 ±0.32
Citrus aurantifolia 400	13.56 ± 0.22*	13.68 ± 0.78	14.44 ±0.54*	30.22 ±0.34
Citrus aurantifolia 600	15.41 ± 0.77*	16.79± 0.45*	16.89 ±0.32*	33.22± 0.21*
Warfarin 0.54mg/kg	16.42±1.10*	16.22±1.1*	15. 03±0.69*	33.15±1.5**

n= 10
Average values ± S.E.M
*P< 0.05 as compared to control
**P< 0.01 as compared to control"

Figure 1: COAGULATION PARAMETERS AFTER 30 DAYS



All values are presented as the mean ± S.D (Standard deviation). The statistical examination was implemented by using one-way ANOVA followed by post hoc Tukey's test for multiple comparisons.

Table 3 presents the effects of A.fistulosum and C.aurantifolia swingle leaves administration on various parameters, including TT, PT, aPTT, and Fg, over a 45-day period in animals.

Animals receiving A.fistulosum extract at a dose of 200mg/kg body weight displayed minimal changes in all parameters compared to the control group. Specifically, the values for TT, PT, aPTT, and Fg were 11.24±0.33, 13.35±0.35, 10.21±0.4, and 30.34±0.37, respectively, while the control animals exhibited values of 9.49±0.70, 11.20±0.49, 10.20±0.64, and 26.6±1.1, respectively.

In contrast, animals administered A.fistulosum extract at a dose of 400mg/kg body weight displayed a significant increase in TT, PT, and aPTT, with values of 16.25±0.22, 16.40±0.55, and 19.45±0.65, respectively. However, the increase in Fg was negligible (31.27±0.34) compared to the control group.

Animals treated with A.fistulosum extract at a dosage of 600mg/kg body weight showed a noteworthy increase in PT (21.89±0.45) and a highly significant increase in TT and aPTT (24.65±0.72 and 35.35±3.2, respectively). Nonetheless, the increase in Fg was inconsequential (33.45±0.32) compared to the control group.

Regarding the administration of C.aurantifolia swingle leaves extract, animals that received a dosage of 200mg/kg body weight showed no significant variation in all parameters (TT, PT, aPTT, and Fg) compared to the control group. Their values were 13.45±0.30, 14.54±0.54, 10.76±0.27, and 32.23±1.24, respectively.

Animals that received a dosage of 400mg/kg body weight of C.aurantifolia swingle leaves extract exhibited a noteworthy increase in TT, PT, and aPTT (18.65±0.43, 18.65±0.2, and 21.45±0.90, respectively), while the increase in Fg was inconsequential (35.4±10.90) compared to the control group.

Animals administered a dosage of 600mg/kg body weight of C.aurantifolia swingle leaves extract showed a noteworthy increase in TT, PT, and Fg (26.23±0.25, 25.34±0.78, and 49.34±1.23, respectively) compared to the control group and a highly significant increase in aPTT (37.65±0.56).

Animals that were given warfarin at a dosage of 0.54 mg/kg body weight exhibited a highly significant increase in all parameters (TT, PT, aPTT, and Fg). Their values were 23.40±1.10, 25.34±0.56, 37.56±3.0, and 55.12±0.56, respectively.

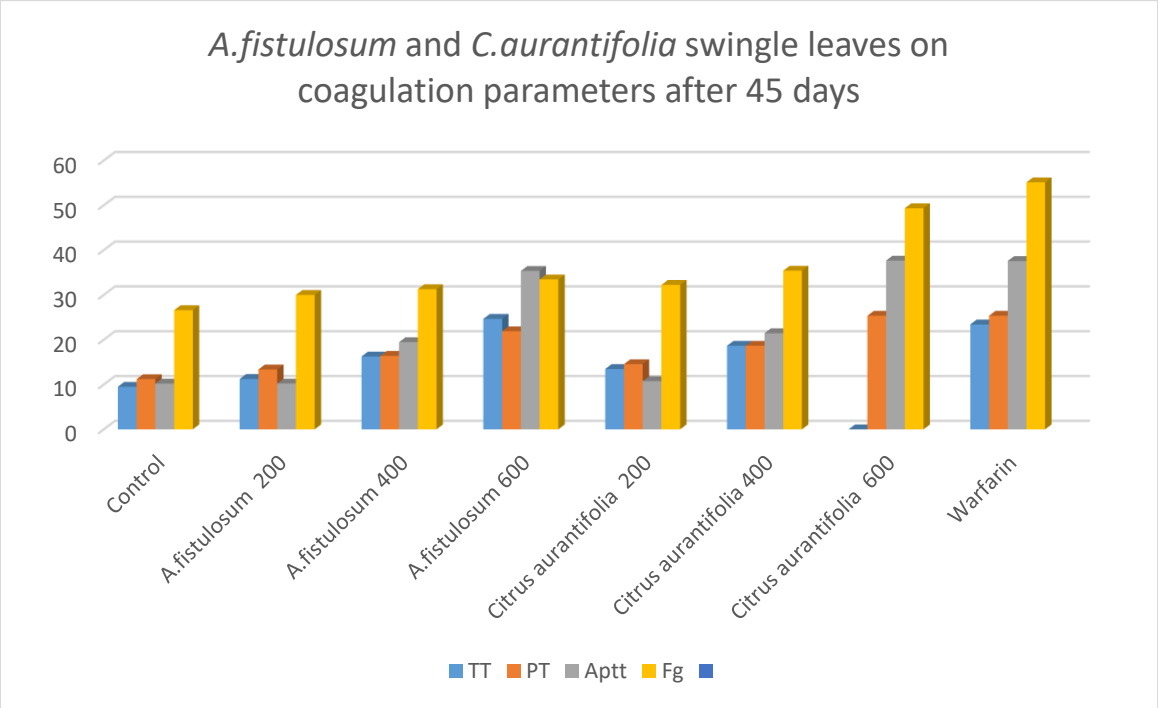
Table 3: Impact of *A. fistulosum* and *C. aurantifolia* Swingle leaves on coagulation parameters after a 45-day duration.

Parameters	TT	PT	aPTT	Fg
Control	9.481±0.70	11.211± 0.49	10.212 ± 0.64	26.62± 1.1
***A.fistulosum* 200**	11.252±0.33	13.322±0.35	10.211±0.4	30.323±0.37
***A.fistulosum* 400**	16.262±0.22*	16.431±0.55*	19.471±0.65*	31.243±0.34
***A.fistulosum* 600**	24.642±0.72**	21.882±0.45*	35.323±3.2**	33.412±0.32
Citrus aurantifolia 200	13.465 ± 0.30	14.533± 0.54	10.771 ± 0.27	32.212 ± 1.24
Citrus aurantifolia 400	18.644 ± 0.43*	18.632 ± 0.2*	21.46 1± 0.90*	35.52 ± 10.90

Citrus aurantifolia 600	26. 221± 0.25*	25.351 ± 0.78*	37.641 ± 0.56**	49.334 ± 1.23*
Warfarin	23.411±1.10**	25.352±0.56**	37.572±3.0**	55.123±0.56**

"n= 10
Average values ± S.E.M
*P< 0.05 as compared to control
**P< 0.01 as compared to control"

Figure 2: COAGULATION PARAMETERS



All values are presented as the mean ± S.D (Standard deviation). The statistical examination was implemented by using one-way ANOVA followed by post hoc tukey's test for multiple comparisons".

DISCUSSION:

Plants have been historically utilized for various purposes, including medicinal treatments, housing, food, clothing, and more. They have played a significant role in traditional medical systems that have existed for thousands of years. A considerable proportion of widely used medications today are derived from plant compounds(8). These natural substances have demonstrated therapeutic effects in treating a wide range of disorders such as cancer, constipation, edema, heart failure, inflammation, pain, and more. Some well-known medicinal plants include red pepper, jaborandi, pacific yew, sweet clover, and opium. The demand for botanical medicines has increased, with retail sales in the United States nearing $4 billion in 1998(8).

Despite the expansion of the botanicals industry, it is important to note that herbal medicines are not subjected to the same stringent regulations as pharmaceutical companies. Inadequate dissemination of information and lax regulation can contribute to the improper use of herbal medicines, which in turn can lead to potentially harmful adverse reactions (9). Limited data is available on the therapeutic efficacy and pharmacological properties of these products, and reports describing their side effects are scarce. Further research is needed to evaluate their clinical value and potential harm

The primary objective of this study was to evaluate the immediate toxicity and therapeutic potential of ethanol extracts derived from the leaves of *Citrus aurantifolia* Swingle and Allium fistulosum. Previous research has already recognized the existence of essential oils in citrus leaves, and a comprehensive phytochemical examination of *Citrus aurantifolia* Swingle leaves has confirmed the presence of a diverse array of compounds, encompassing flavonoids, alkaloids, saponins, glycosides, phenols, steroids, tannins, and terpenoids (10). These ingredients may contribute to the hypoglycemic activity observed in the study.

The administration of extracts derived from *A. fistulosum* and *Citrus aurantifolia* Swingle leaves at doses of 400mg/kg and 600mg/kg resulted in significant improvements in Thrombin time and Activated partial thromboplastin time in the treated animals. Animals receiving the extracts at 400mg/kg also showed a notable increase in Prothrombin time. These experiments demonstrated the extracts' ability to exert anticoagulant effects by inhibiting the coagulation cascades. It is believed that flavonoid compounds and total polyphenols are the active components responsible for these observed activities. Flavanols play a role in scavenging oxygen-free radicals and act as antithrombotic agents by maintaining prostacyclin and nitric oxide levels. Additionally, flavonoids exhibit antithrombotic properties by blocking the cyclooxygenase and lipoxygenase pathways.

The study revealed a significant extension in the activated partial thromboplastin time, indicating a deficiency in critical coagulation factors within the intrinsic pathway. It is suggested that the leaves of *A. fistulosum* and *Citrus aurantifolia* Swingle may employ a mechanism akin to heparin by forming complexes with antithrombin III. Furthermore, the research demonstrated a marked increase in thrombin time, suggesting the inhibition of thrombin due to reduced coagulation factor activity. Chlorogenic acid, found in the extracts, can break down fibrin clots and inhibit procoagulant proteases, resulting in prolonged coagulation parameters. Cholinergic acid, present in *A. fistulosum*, is believed to contribute to its anticoagulant activity.

CONCLUSION:

In conclusion, plants remain a valuable reservoir of medicinal compounds. Nevertheless, to guarantee the secure and efficient utilization of herbal medicines, it is imperative to implement adequate regulations and conduct further research. This study has illustrated both the acute toxicity and therapeutic benefits of ethanol extracts derived from *Citrus aurantifolia* Swingle leaves and

Allium fistulosum. It has shed light on their potential anticoagulant properties and has offered valuable insights into the active constituents responsible for these effects.

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