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Experimental Demonstration of Blood Coagulating Property of *Tagetes Patula* & *Atriplex Hortensis Var.Rubra*

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Abstract: **Marigold (*Tagetes patula*) and Red orach(*Atriplex hortensis var.rubra*) besides being ornamental plants, have various medicinal properties—they are nematocidal,fungicidal, antibacterial and insecticidal and aids in wound healing. Extraction of active constituents was done by conventional as well as the Soxhlet method, which was found to be much more efficient using a petroleum ether (B.P 60̊) as solvent. Blood clotting activity of the leaf extract were examined using aminal models (Rabbits and Mice) invivo for both topical and systemic administration .Our work is focused on the blood clotting activity of the leaf extracts and to check the type of chemically active ingredients present in them by various phytochemical evaluation.Analytical methods like Thin Layer Chromatography and Ultraviolet Visible Spectroscopy was also performed for qualitative analysis and comparison with a standard drug. Further research is needed for identification and quantification of its bioactive compounds, which could be purified further and chemically isolated .Further improvisation on this can possibly make it a suitable medication on use with appropriate vehicle.**

***Keywords*: *Tagetes patula*(Marigold);*Atriplex hortensis var.rubra*(Red orach);blood clotting activity,Thin Layer chromatography,Ultraviolet Visible Spectroscopy.**

1. INTRODUCTION

Tagetes patula, also known as marigold, is a popular ornamental plant and grows in various agroclimates. Beside its use as an ornamental plant, it is also cultivated as a crop. The species *Tagetes minuta, Tagetes erecta, and Tagetes tenuifolia* are the most common in tropical countries. Bioactive extracts from different parts of this plant exhibit nematocidal, fungicidal and insecticidal activities. Marigold leaf extracts have also shown antibacterial property against several gram-positive and gram-negative bacteria. It also has antioxidant and wound healing properties. Research is still in progress to find the possible mechanism of action for the wound healing property of marigold leaves. Another plant known as Red Orach(*Atriplex hortensis var.rubra*) was also studied for the same purpose.Coagulation of blood after vascular spasm and platelet aggregation involves formation of prothrombinase and conversion of prothrombin into thrombin, which helps in conversion of fibrinogen into fibrin (clot). In the present study, *Tagetes patula* leaf extracts and *Atriplex hortensis var.rubra* extracts were obtained from two different extraction methods (conventional and Soxhlet) and comparative analysis of yield percentages as well as blood coagulation activity (in terms of bleeding time), was performed on test animals-Rabbits and Mice(Biological evaluation).

1.1 Materials and methods:

- Marigold (*Tagetes paluta*) leaves and Red Orach(*Atriplex hortensis var rubra*) were collected from a local plant nursery, Durgapur, India. All standard chemicals were obtained from the various chemical units E. Merck India Ltd., HiMedia Private Ltd.. (India).

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- Processing of the leaves-Fresh marigold leaves and red orach were chopped and subjected to drying in a shade in a static environment at a temperature of 40 ±5 °C for 7 days. The leaves were then crushed to a powder form using an electric grinder and stored in an airtight glass bottle at refrigerated temperature (4 °C) for further analysis.
- Conventional(Percolation) extraction- Of the powdered leaves, 5.00±0.05 g from each type of leaves was taken in a conical flask seperately, and 50 ml of solvent was added and kept in percolator for 2 days.This extraction process was carried out using three different types of solvents-water, pertroleum ether(60℃-80℃) and ethanol.
- Soxhlet extraction-An amount of 5.00±0.05 g of powdered marigold leaves and red orach leaves were placed in a Whatman cellulose thimble (25 mm×60 mm).Samples were extracted in a Soxhlet extraction system using 100 ml of different solvents. The heating power was set at two cycles per hour so that six cycles of extraction was achieved within 3 h. As with the conventional extraction, this process was carried out using three different solvents—water, petroleum ether (60℃-80℃) and ethanol.

1.2 Exclusion of solvent:

The crude extract solutions obtained were then filtered using Whatman filter paper and concentrated using a vacuum rotary evaporator (BUCHI Rotavapor Model R-210at 60 °C or lower to remove the ethanol, lyophilized to remove waterand dried using a freeze dryer (LT5S, Lyophilization Systems,Inc., USA, with five shelves of 0.9 m2). The lyophilizer was used to reduce the temperature down to −30 °C, and the coolants used were R404A (44 % w/w pentafluoroethane,52 % w/w 1,1,1-trifluoroethane, 4 % w/w 1,1,1,2 tetrafluoroethane) and R508R (54 % w/w hexafluoroethane,46 % w/w trifluoroethane) at 150 and 200 psi, respectively.Pressure was reduced to 3.3×10−4 Pa for primary drying and 3.3×10−5 Pa for secondary drying.Heat was supplied and maintained at 25℃ to sublime ice into vapour.After 16 hours of drying ,the samples were collected and stored at 4℃ for further analysis.The petroleum ether extracts were dried at room temperature as petroleum ether is a highly volatile liquid.

Table 1: Comparative yield percentage of marigold leaf by conventional and Soxhlet extraction methods with different solvents.

Solution no	Solvent	% extracted by conventional method	% extracted by soxhlet apparatus method
1 2 3	Water Ethanol Petroleum ether	3.23%± 0.06 4.34%±0.04 5.13%±0.05	4.16%±0.05 5.89%±0.08 6.48%±0.07
		Maximum yield with pet. ether	Maximum yield with pet.ether

Table 2: Comparative yield percentage of Red orach leaf by conventional and Soxhlet extraction methods with different solvents.

Solution no	Solvent	% extracted by conventional method	% extracted by soxhlet apparatus method
 2 3	Water Ethanol Petroleum ether	4.83%± 0.05 5.34%±0.06 7.13%±0.02	5.50%±0.05 6.16%±0.08 8.78%±0.07
		Maximum yield with pet.ether	Maximum yield with pet.ether

1.3 Datas obtained from chart and inference:

The yield percentages of the marigold leaf extract using the conventionalextraction method with different solvent systems (water,ethanol and petroleum ether) were3.23%± 0.06,4.34%±0.04 and 5.13%±0.05 respectively(TABLE 1). Comparatively, the Soxhlet method yield percentages were 4.83%± 0.05, 5.34%±0.06 and 7.13%±0.02 respectively (TABLE 2). Based on these data, it can be concluded that the Soxhlet extraction is suitable for bioactivity analysis since it has greater yield than the conventional method of extraction.

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1.4 Phytochemical Investigation:

Phytochemical tests are done to check the type or class of chemical components present in the leaf extracts.For carrying out the test various reagents are used. Change in colour is the main confirmatory test behind phytochemical study.The various phytochemical test performed are mentioned in the TABLE given below with visual results in TABLE 3.

Table 3: Table showing different chemical tests performed and their results

Name of the test with description	Active chemical group present	*Tagetes patula*	*Atriplex hortensis var rubra*
Molisch's test-To the filtrate few drops of alcohol alpha naphthol and 2ml of conc.sulphuric acid was added slowly by the side of the test tube.A violet coloured ring appears at the junction of two layers.	Carbohydrates	Absent	Absent
Salkowski's test- To the extract few drops of conc.sulphuric acid was added A red colouration at lower layer Yellow colouration at lower layer	Steroids	Present	Present
Borntrager's test- A little liquid extract was treated with chloroform,chloroform layer was decanted off and equal quantity of dilute ammonia solution was added.A pink colour is produced in the ammoniacal layer	Terpenoids Glycoside	Absent Absent	Absent Absent
Ninhydrin test- The liquid extract was treated with Ninhydrin test reagent.Blue colour produced shows the presence of amino acids			
Shinoda's test-Small quantity of extract was dissolved in alcohol and to that pieces of magnesium was added followed by conc.hydrochloric acid dropwise and heated Presence of magenta colour is seen	Amino Acid Flavonoids	Absent Absent	Absent Absent

2. ANALYTICAL PROCESS 1 -THIN LAYER CHROMATOGRAPHY

Thin-layer chromatography (TLC) is a chromatography technique used to separate non-volatile mixtures. Thin-layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose. This layer of adsorbent is known as the stationary phase.After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved.

For Stationary phase Silica Gel GF was used.

Mobile phase used was a combination of benzene:chloroform:ethyl alcohol:methanol in the ratio of 4:3:2:1.

Technique-The process is similar to paper chromatography with the advantage of faster runs, better separations, and the choice between different stationary phases. Because of its simplicity and speed TLC is often used for monitoring chemical reactions and for the qualitative analysis of reaction products.

To run a thin layer chromatography, the following procedure is carried out:

- A small spot of solution containing the sample is applied to a plate, about 1.5 centimeters from the bottom edge. The solvent is allowed to completely evaporate off, otherwise a very poor or no separation will be achieved. If a non-volatile solvent was used to apply the sample, the plate needs to be dried in a vacuum chamber.

- A small amount of an appropriate solvent (eluent) is poured into a glass beaker or any other suitable transparent container (separation chamber) to a depth of less than 1 centimeter. The container is closed with a cover glass or any other lid and is left for a few minutes to let the solvent vapors ascend and saturate the air in the chamber.
- The TLC plate is then placed in the chamber so that the spot(s) of the sample do not touch the surface of the eluent in the chamber, and the lid is closed. The solvent moves up the plate by capillary action, meets the sample mixture and carries it up the plate (elutes the sample). The plate should be removed from the chamber and dried.

2.1 Separation Process and Principle:

Separation of compounds is based on the competition of the solute and the mobile phase for binding places on the stationary phase. For instance, if normal-phase silica gel is used as the stationary phase, it can be considered polar. Given two compounds that differ in polarity, the more polar compound has a stronger interaction with the silica and is, therefore, more capable to dispel the mobile phase from the binding places. As a consequence, the less polar compound moves higher up the plate (resulting in a higher R_f value). If the mobile phase is changed to a more polar solvent or mixture of solvents, it is more capable of dispelling solutes from the silica binding places, and all compounds on the TLC plate will move higher up the plate. It is commonly said that "strong" solvents (eluents) push the analyzed compounds up the plate, whereas "weak" eluents barely move them. The order of strength/weakness depends on the coating (stationary phase) of the TLC plate.

2.2 Analysis:

As the chemicals being separated may be colorless, several methods exist to visualize the spots:

When silica Gel GF is used as an adsorbant it allows the visualization of spots under UV light (254 nm). The adsorbent layer will thus fluoresce light-green by itself, but spots of analyte quench this fluorescence.

Iodine vapors are a general unspecific color reagent.

Once visible, the R_f value, or retardation factor, of each spot can be determined by dividing the distance the product traveled by the distance the solvent front traveled using the initial spotting site as reference. These values depend on the solvent used and the type of TLC plate and are not physical constants.

Fig.1: From left Atriplex hortensis var.rubra, Standard drug (Vit K1) phytomenadione, Tagetes patula,vehicle (light liquid paraffin).

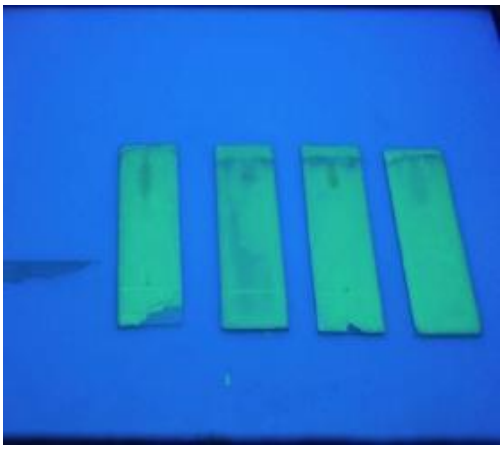

Fig.2: From left Tagetes patula, Standard drug (Vit K1) phytomenadione, Atriplex hortensis var.rubra, vehicle (light liquid paraffin) under UV light (254nm)

2.3 Results:

Rf(Retardation Factor) is calculated by the formula:-

Rf=Distance travelled by analyte molecule/Distance travelled by solvent front.

Rf(for Standard drug phytomenadione injection BP 1mg/0.5ml)=4.5/5.7=**0.78.**

Rf(for Marigold)=4.5/5.8=**0.77.**

Rf(for Red orach)=4.5/5.7=**0.78.**

2.4 Interpretation from obtained data:

It was calculated and found that Rf(Retardation factor) value of standard drug (phytomenadione injection BP 1mg/0.5ml) ie. 0.78 is close to that of Rf value of Marigold(0.77) and Rf (Retardation factor) value of Red Orach(0.78).It can thus be assumed that the extracts contain same active constituent under study as that of the standard drug as the Rf values are near about same.

3. ANALYTICAL PROCESS II- ULTRAVIOLET VISIBLE SPECTROSCOPIC ANALYSIS

Ultraviolet (UV) and visible absorption techniques encompass analytical methods based upon measurement of light absorption by substances in the wavelength region from 190-900 nm. The region from 190 to 380 nm is known as the UV region. And from 380 – 900 nm, the visible region of the spectrum Absorption in the UC – visible region arises from electronic transitions within the molecule. A variety of instruments are available for measuring light absorption in the UV- visible region of the spectrum. In a single beam instrument, light passes through a monochromator and then through the sample and into the detector.

3.1 Materials required:

Concentrated stock standard, Distilled or deionized water for dilution, Instrument for accurately measuring small volumes of liquid (ex. Pipette), Graduated cylinders, beakers, or volumetric flasks (appropriately sized for desired volume of solutions

3.2 Preparation of standard solution containing standard drug (phytomenadione):

To 2mg/ml of standard drug 99ml distilled water was added to make volume upto 100ml

Then again 1ml of stock solution was taken for serial dilution with 9ml distilled water

Since 1µg/ml= 1ppm

Therefore 1mg/ml=1000ppm

2mg/ml=2000µg/ml=2000ppm

We make up the volume upto 100ml with 1ml standard drug and 99ml distilled water=200ppm

Then 1ml from stock solution was withdrawn and volume made upto 9ml=20ppm

3.3 Preparation of test drug (extract) solution using acetone as solvent:

Since we do not know the actual concentration of the analyte present in the extract and the extract contains other components as well we take an approximate 1mg of it in extract and dissolve it in 1ml of acetone.From there we make-up the volume upto 100ml with 99ml acetone.From this solution we take 2ml of drug solution and dissolve it in 18ml acetone upto 20ml.

Since 1µg/ml= 1ppm

Therefore 1mg/ml=1000ppm

1ml of stock solution was made upto 100ml with 99ml acetone=100ppm

From previous solution 2ml was taken and volume made upto 20ml with 18ml acetone=20ppm

3.4 Ultraviolet Visible Spectroscopic Analysis:

For standard drug: Distilled water was taken as blank in a cuvette and UV spectroscopy was run for wavelengths ranging from 380nm-800nm .Distilled water was taken as blank since distilled water was used to dissolve the test drugs so as to understand the effects of solvent on spectra of drugs analysed.

For Test(extracts):Acetone was taken as blank in a cuvette and UV spectroscopy was run for wavelengths ranging from 380nm-800nm .Acetone was taken as blanl since acetone was used to dissolve the test drugs so as to understand the effects of solvent on spectra of drugs analysed.

Graph with Absorbance Vs Wavelength was obtained for all wavelengths ranging from 190nm to 700nm which denotes the Ultraviolet visible range.

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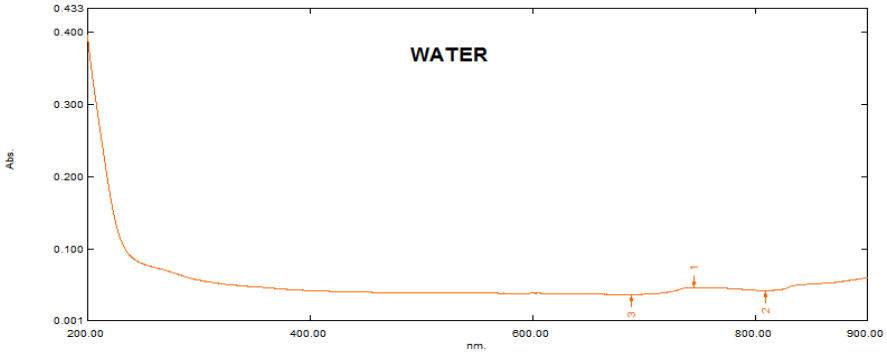
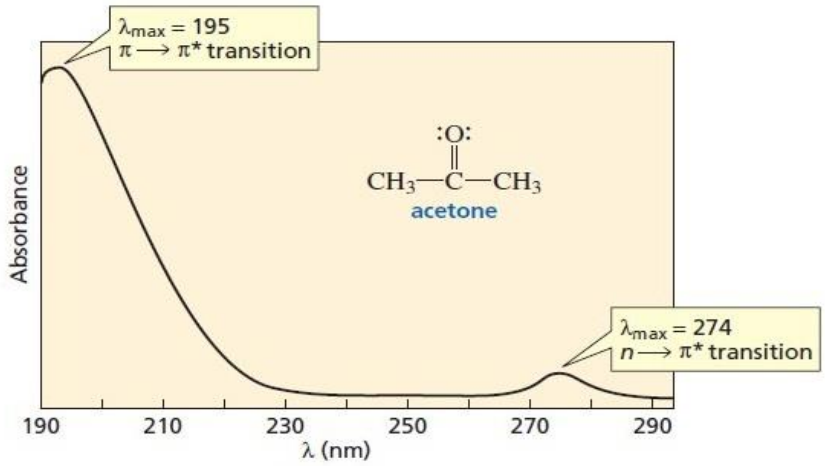
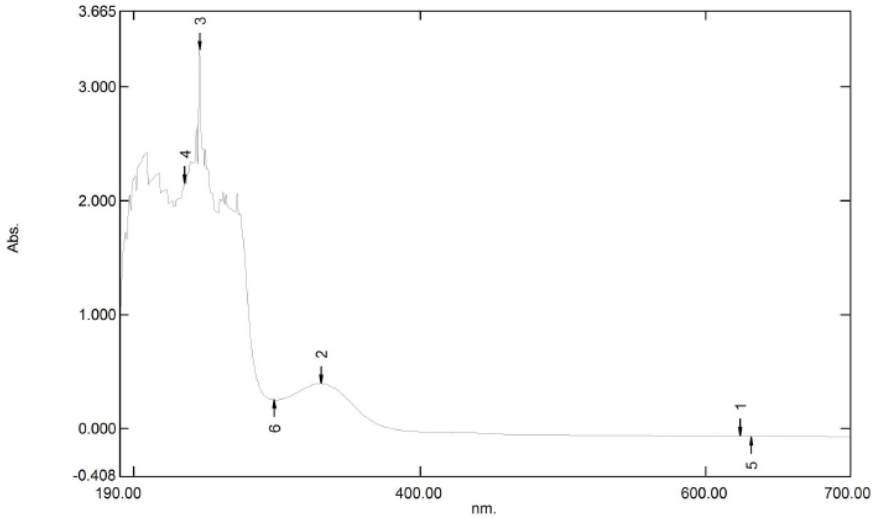


Graph 1: Distilled Water spectral analysis
UV-Vis Spectral analysis data is to be observed to nullify its effect as solvent.



Graph 2: Acetone spectral analysis
UV-Vis Spectral analysis data is to be observed to nullify its effect as solvent.



Graph 3 – Spectrophotometric peak diagram for standard drug (Phytomenadione)

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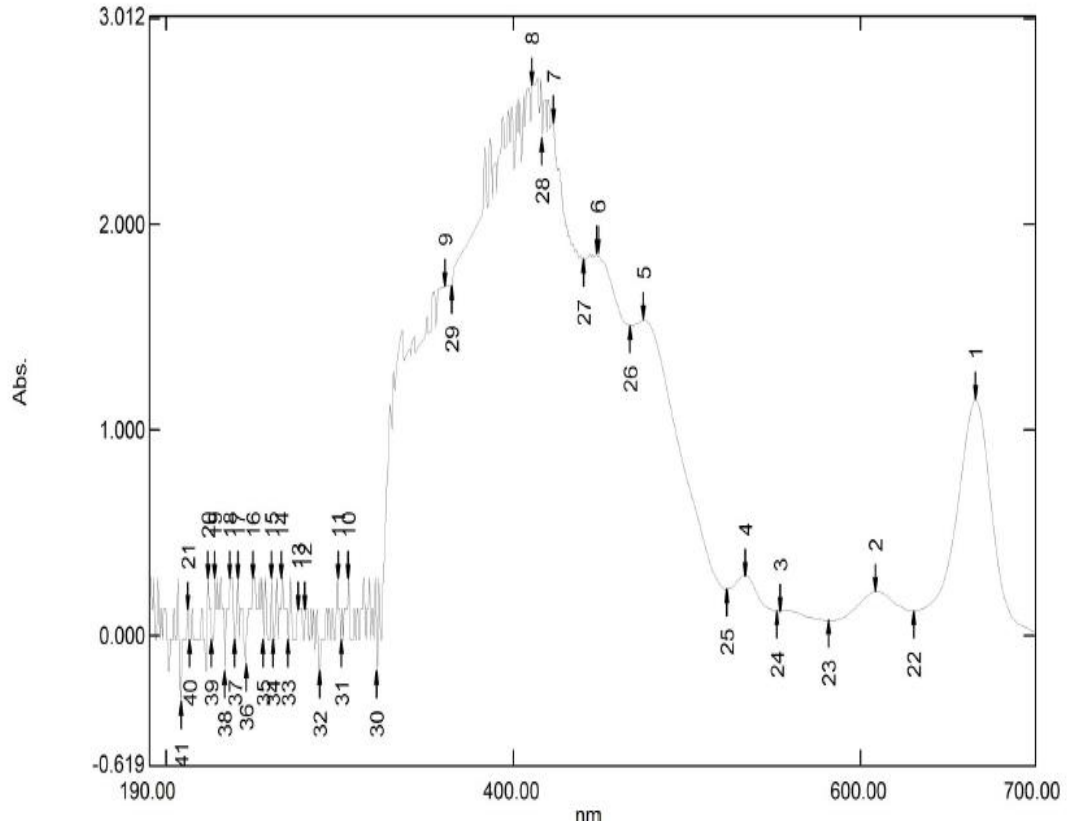
Measurement Properties	
Wavelength Range (nm.):	190.00 to 700.00
Scan Speed:	Medium
Sampling Interval:	0.5
Auto Sampling Interval:	Enabled
Scan Mode:	Auto
Sample Preparation Properties	
Weight:	1
Volume:	4
Dilution:	10
Path Length:	420
Additional Information:	asd
Instrument Properties	
Instrument Type:	UV-1700 Series
Measuring Mode:	Absorbance
Slit Width:	1.0 nm
Light Source Change Wavelength:	340.8 nm
S/R Exchange:	Normal
Attachment Properties	
Attachment:	None

TABLE 4: Table showing Wavelength vs Absorbance of

No.	P/V	Wavelength	Abs.	Description
1	↑	623.50	-0.063	
2	↑	330.50	0.401	
3	↑	245.50	3.325	
4	↑	235.00	2.157	
5	↓	632.00	-0.066	
6	↓	298.00	0.254	



Graph 4 – Spectrophotometric peak diagram for test drug extract (*Tagetes patula*)

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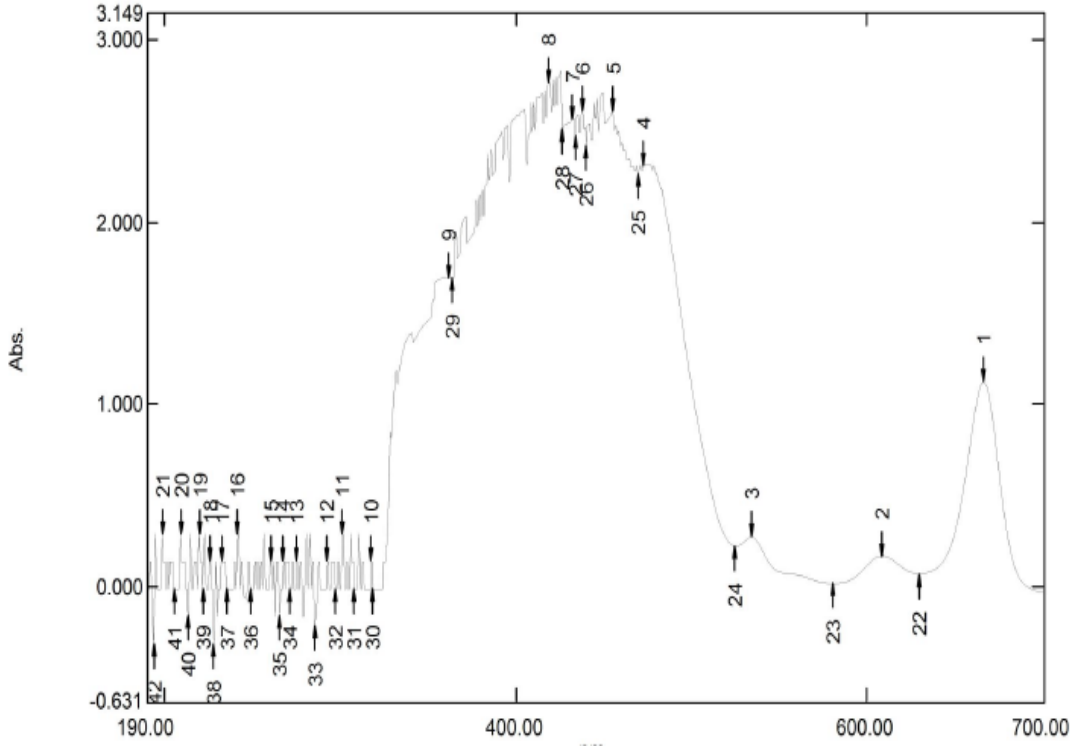
TABLE 5 (a): Table showing Wavelength vs Absorbance of *Tagetes*

Measurement Properties	
Wavelength Range (nm.):	190.00 to 700.00
Scan Speed:	Medium
Sampling Interval:	0.5
Auto Sampling Interval:	Enabled
Scan Mode:	Auto
Sample Preparation Properties	
Weight:	1
Volume:	4
Dilution:	10
Path Length:	420
Additional Information:	asd
Instrument Properties	
Instrument Type:	UV-1700 Series
Measuring Mode:	Absorbance
Slit Width:	1.0 nm
Light Source Change Wavelength:	340.8 nm
S/R Exchange:	Normal
Attachment Properties	
Attachment:	None

No.	P/V	Wavelength	Abs.	Description
1	↑	666.00	1.151	
2	↑	608.50	0.220	
3	↑	553.00	0.126	
4	↑	533.00	0.299	
5	↑	474.00	1.537	
6	↑	448.50	1.858	
7	↑	423.00	2.498	
8	↑	411.00	2.683	
9	↑	360.50	1.699	
10	↑	305.00	0.286	
11	↑	298.50	0.286	
12	↑	280.00	0.135	
13	↑	275.50	0.135	
14	↑	266.50	0.286	
15	↑	260.50	0.286	
16	↑	249.50	0.286	
17	↑	241.00	0.286	
18	↑	236.50	0.286	
19	↑	228.00	0.286	
20	↑	224.00	0.286	
21	↑	212.00	0.135	
22	↓	631.00	0.124	
23	↓	581.50	0.075	
24	↓	551.50	0.126	



Graph 5 – Spectrophotometric peak diagram for test drug extract (*Atriplex hortensis var.rubra*)

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TABLE 5 (b): Table showing Wavelength vs Absorbance of *Atriplex hortensis*

Measurement Properties	
Wavelength Range (nm.):	190.00 to 700.00
Scan Speed:	Medium
Sampling Interval:	0.5
Auto Sampling Interval:	Enabled
Scan Mode:	Auto
Sample Preparation Properties	
Weight:	1
Volume:	4
Dilution:	10
Path Length:	420
Additional Information:	asd
Instrument Properties	
Instrument Type:	UV-1700 Series
Measuring Mode:	Absorbance
Slit Width:	1.0 nm
Light Source Change Wavelength:	340.8 nm
S/R Exchange:	Normal
Attachment Properties	
Attachment:	None

No.	P/V	Wavelength	Abs.	Description
1	↑	666.00	1.120	
2	↑	608.50	0.166	
3	↑	534.00	0.269	
4	↑	472.50	2.312	
5	↑	455.00	2.613	
6	↑	438.00	2.613	
7	↑	432.00	2.571	
8	↑	418.50	2.767	
9	↑	361.50	1.699	
10	↑	317.50	0.135	
11	↑	301.00	0.286	
12	↑	292.50	0.135	
13	↑	275.00	0.135	
14	↑	267.50	0.135	
15	↑	260.00	0.135	
16	↑	241.50	0.286	
17	↑	232.00	0.135	
18	↑	225.50	0.135	
19	↑	219.50	0.286	
20	↑	209.00	0.286	
21	↑	198.50	0.286	
22	↓	629.50	0.068	
23	↓	580.00	0.013	
24	↓	525.00	0.219	

3.5 Inference and discussions:

UV spectrophotometers measure the visible regions of ultraviolet light and can provide valuable information about the levels of active ingredients present in pharmaceutical compounds, as well as detect any impurities. By measuring the absorption of UV radiation of light, spectrophotometric analysis can quantify these levels at a highly accurate rate.Total spectral analysis showed many peaks in crude extracts indicating presence of multiple ingredients/ chemicals present in the tested extract. Fewer peaks are observed when extracts are purified for isolation of specific chemicals. UV-Vis Spectroscopy is beneficial in qualitative analysis as we can get spectra with specific solvent extraction and dissolving in specific solvent.

From the spectral peak report the following conclusions can be drawn:-

- ✓ A number of peaks were observed for both the test drugs due to presence of numerous chemical moieties.
- ✓ The standard drug(vitamin K)showed an upward peak at wavelength 330.50 nm with an absorbance value of 0.401.
- ✓ The test drug(*Tagetes Patula*) showed an upward peak at wavelength 301nm with an absorbance value of 0.286.The test drug contain a number of compounds as well as impurities.Vitamin K might also be present in combined form with other substances.
- ✓ The test drug(*Atriplex hortensis var rubra*) shows an upward peak at wavelength 305.00nm with an absorbance value of 0.286.The test drugs contain a number of compounds as well as impurities. Vitamin K might also be present in combined form with other substances.
- ✓ From these spectral report it can be concluded that blood clotting activity is present in both the test extract(either in free or combined form).
- ✓ Further isolation and purification of vitamin K from test drugs and their spectrophotometric analysis will give more satisfactory results.

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4. ANIMAL STUDY I

4.1 Aim: To determine the bleeding time in rabbits through topical administration of drugs

Theory – Bleeding time is a medical test done on a subject to assess his platelets function. It involves making a subject bleed then timing how long it takes for them to stop bleeding.

Duke Method-With the Duke method, the patient is pricked with a special needle or lancet, preferably on the earlobe or fingertip, after having been swabbed with alcohol. The prick is about 3–4 mm deep. The blood is wiped every 30 seconds with a filter paper. The test ceases when bleeding ceases. For humans the usual time is about 2–5 minutes. In the present study similar test is done on the earlobe of an adult rabbit.

4.2 Materials taken:

15 rabbits, standard drug, 2 extracts from plants (test), Light liquid paraffin, syringes and needles, cotton, ethyl alcohol, antiseptic.

4.3 Procedure:

1. For standard drug (phytomenadione):- 1mg/0.5ml standard drug was used without any dilution.

2. For extracts (test drugs):- 1mg of extract drugs were accurately weighed and were dispersed in 0.5ml of vegetable oil.They were then evenly mixed with the help of a spatula.

Administration of standard and test drugs on animals:-

Ethanol was used for dilation of blood vessels of the earlobe of rabbits.

For rabbits – 5 groups (according to drugs and vehicle administered).

1st group- no drug (control).

2nd group- vehicle used is light liquid paraffin.

3rd group – standard drug phytomenadione.

4th group- extract of test drug 1 + vehicle(light liquid paraffin).

5th group- extract of test drug 2 + vehicle(light liquid paraffin).

Incision was made in the ear vein with the help of needle till bleeding starts.Then bleeding time was noted for each animal under study with the above mentioned drugs & vehicle.

TABLE 6: The following results were obtained for each group:

Group no.	Drug/vehicle under study	Bleeding time(in seconds)	Average of bleeding time(in seconds)
I	BLANK(CONTROL)	51.23 49.35 46.56	49.04
II	VEHICLE CONTROL(LIGHT LIQUID PARAFFIN)	50.24 48.34 47.23	48.60
III	STANDARD DRUG(PHYTOMENADIONE BP)	31.13 29.27 36.47	32.29
IV	EXTRACT-TEST DRUG 1 (*Tagetes patula*)	35.11 41.24 42.27	39.54
V	EXTRACT-TEST DRUG 2 (*Atriplex hortensis var. rubra*)	43.54 33.23 35.53	37.50

4.4 Inference and discussons:

From the above chart we can conclude that the extract (test) drugs have a property to decrease the bleeding time. The average bleeding time for blank was found to be 49.04 seconds whereas the average bleeding time after topical administration of standard vitamin K was found to be 32.29 seconds.The vehicle used(Light liquid paraffin) has no effect on bleeding time.The same prodecure was carried out with the extracts of *Tagetes patula* & *Atriplex hortensis var rubra* and bleeding time on average was found to be 39.54 seconds and 37.50 secondss respectively. This proves that the test drugs also posses blood coagulation property like that of vitamin K.Further isolation and identification will provide more promising results.

5. ANIMAL STUDY II

5.1 Aim:

To determine the bleeding time in mice through intra-peritoneal administration of drugs

5.2 Theory:

Bleeding time is a medical test done on a subject to assess his platelets function. It involves making a subject bleed then timing how long it takes for them to stop bleeding.In the present study the tail vein of mice were punctured to make them bleed and bleeding time was recorded by wiping away the blood using filter paper .The time when the bleeding completely ceases denotes the bleeding time of the subject. This test is carried out after intra-peritoneal administration of test drugs and standard drug and comparing their values.

Materials taken- 30 mice, standard drug, 2 extracts from plants (test), Light liquid paraffin, syringes and needles, cotton , ethyl alcohol, blade, antiseptic.

5.3 Procedure:

1.Calculation for standard drug (phytomenadione):-

From literature the normal dose of vitamin K for an infant of average weight 3kg is 500mcg – 1000 mcg.

So, taking an average of 750 mcg for 3kg infant

From above the average dose / kg is 250 mcg

1 kg human-→ 250 mcg of drug

20gm mouse→ (250 x 20) / 1000 = 5 mcg.

The standard drug has concentration of 1 mg / 0.5 ml

1mg = 1000 mcg

1000 mcg drug is present in 0.5 ml

So, the resulting diluton contains 5 mcg drug

5 mcg→ (0.5ml x 5)/1000 = 0.0025ml standard drug was administered to each mouse.

2.Calculations for extracts(test) :- As we do not know the actual concentration of the active constituent and other components too present along with it .Therefore we take 2mg of extract(test) drug and dissolve it in 1ml of liquid paraffin which gives the concentration of 2mg/1ml.

3. Administration of standard and test drugs on animals :-Ethanol was used for dilation of blood vessels of the earlobe of rabbits.

For rabbits – 5 groups (according to drugs and vehicle administered).

1st group- no drug (control).

2nd group- vehicle used is light liquid paraffin.

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3rd group – standard drug phytomenadione.

4th group- extract of test drug 1 + vehicle(light liquid paraffin).

5th group- extract of test drug 2 + vehicle(light liquid paraffin).

Incision was made in the ear vein with the help of needle till bleeding starts.Then bleeding time was noted for each animal with the above mentioned drugs & vehicle.

TABLE 7: The following results were obtained for each group:

Group no.	Drug/vehicle under study	Bleeding time(in seconds)	Average of bleeding time(in seconds)
I	BLANK(CONTROL)	76.45 78.34 72.54	75.77
II	VEHICLE CONTROL(LIGHT LIQUID PARAFFIN)	71.45 73.36 80.34	75.05
III	STANDARD DRUG(PHYTOMENADIONE BP)	20.23 27.39 26.17	24.59
IV	EXTRACT-TEST DRUG 1 (*Tagetes patula*)	49.23 51.36 52.46	51.01
V	EXTRACT-TEST DRUG 2 (*Atriplex hortensis var. rubra*)	41.25 39.23 38.57	39.68

5.4 Inference and discussions:

From the above chart we can conclude that the extract (test) drugs have a property to decrease the bleeding time. The average bleeding time for blank was found to be 75.77seconds whereas the average bleeding time after intra-peritoneal administration of standard vitamin K was found to be 24.59 seconds. The vehicle (Light liquid paraffin) has no effect on bleeding time. The same procedure was carried out with the extracts of *Tagetes patula* & *Atriplex hortensis var rubra* and bleeding time on average was found to be 51.01seconds and 39.68seconds respectively. This proves that the test drugs also posses blood coagulation property like that of vitamin K. Further isolation and identification will provide more promising results

6. CONCLUSION

The aim of this work was to find out the scientific reasons behind the used of the leaves of *Tagetes patula* and *Atriplex hortensis var rubra* as anti-bleeding agents. These two leaves are used as "ETHNO-BOTANICAL" drugs all over India for stopping external bleeding.Here the work was targeted to justify these points:

1. Whether these leaves can actually reduce the bleeding time?

2. What is the cause behind reduction in bleeding time?

From the experiments the results were found that the bleeding time in rabbits were 49.04 ,32.29,39.54 and 37.50 seconds respectively for control(blank),standard control(with Vitamin K),extracts of *Tagetes patula and Atriplex hortensis var rubra.*

Again the bleeding times in mice were 75.77,24.59,59.01 and 39.68 seconds respectively for control(blank),standard control(with Vitamin K),extracts of *Tagetes patula* and *Atriplex hortensis var rubra*.In both the animals the bleeding times were reduced by both the leaf extracts in comparison to control.

The mechanism of clotting is that when blood is shed,the platelets(coming in contact with rough water wettable surface) disintegrate and liberate thromboplastin.This thromboplastin with the help of calcium ion convert the prothrombin,already present in blood itself to thrombin.Then this thrombin react with fibrinogen that is also present in blood itself to convert fibrinogen to fibrin(clot).For commencing these reactions near about 12 factors are involved in blood.It is also known that the leaves of many plants contain vitamin K.Vitamin K is essential for synthesis of factors II,VII,IX and X.

The powdered leaves of these plants were extracted with petroleum ether as vitamin K is fat-soluble. These two petroleum ether extract were analysed by TLC method(Thin Layer Chromatography) and UV visible spectrophotometric methods and vitamin K was traced out in both the leaf ample extracts. Therefore it may be concluded from this work that the extract of both these leaves can reduce bleeding time and the reason maybe the presence of vitamin K in both the extracts.

Besides these, there might be other factors for which further work on isolation and purification is required.

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