Modulatory effect of *Crassocephalum crepidioides* Benth S. Moore leaf methanol extract and fractions on blood coagulation of Streptozotocin-induced diabetic rats.

By

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OUTLINE

- Background to the Study
- Materials and Methods
- Results
- Conclusion
- Recommendation

BACKGROUND 1/6

- Blood coagulation is an intricate cascade of reactions that involve many proteins (factors) which must act in exact sequence to produce clot formation. The process is rapid and efficient and requires regulation (Karch, 2012).

- A shift in the balance between blood coagulation and inhibition of coagulation to favour either pro- or anticoagulation may result in life-threatening thromboembolism or haemorrhage (Ovanesov, 2015).

Background Cont. 2/6

Diabetes Mellitus

- Diabetes mellitus is a potentially morbid condition characterized by hyperglycemia

- In diabetic state, there is a compromise of the thrombo-haemorrhagic balance that exists in the blood flow of a healthy individual (Nnah, 2015).

- This impairment makes diabetic patients to be susceptible to thromboembolic complications that may lead to aggravation of the diseased state (Ghosh, 2002).

Background cont. 3/6

- Diabetic patients are reported to experience atherosclerosis and more complicated vascular conditions because of increased activation of platelets and coagulation factors, and reduced fibrinolysis (Carr 2001; Grant, 2005).

- This make them more susceptible to plaque rupture and thromboembolism (Fayeza *et al.,* 2015).

Background cont. 4/6

Crassocephalum crepidioides

- *Crassocephalum crepidioides (*fireweed ragleaf) is an annual edible plant that is widespread in tropical and sub tropical regions (Bahar *et al.,* 2017; Rajesh, 2011).

- Local names of the plant:

- Ebolo – Yoruba (Southwest, Nigeria). Adams, 1983.
- Mkpafit – Akwa Ibom (South- south, Nigeria).
- Obuinenawa – Edo (Omotayo *et al.,*2015)
- Gbolo – Benin republic (Adjatin *et al.,* 2013)
- Ye tong hao – Chinese
- Eyukula – Portuguese (Tomimori *et al.,* 2012)

Figure 1: Pictorial view of C. *crepidioides;* wapnus.biorave.org

Background cont. 5/6

Ethnomedical use of C. crepidioides include:

- Treatment of indigestion and headache (Sakpere *et al.*, 2013).
- As laxative *and* purgative, and as a remedy of liver problem (Fowomola & Akindahunsi, 2005; Ayodele, 2007).
- Diarrhea (Rajesh, 2011).
- stomach ulcer (Rajkumari *et al.*, 2013).
- hepatitis, fever and edema (Tomimori *et al.*, 2012; Aniya *et al.*, 2005).
- *Treatment of wound, boils, and burns (Ajibesin, 2012).

Background cont. 6/6

- Scientific reports have revealed its antibiotic, anti-inflammatory, antioxidant, cancer chemopreventive and hypoglycemic activities (Bahar *et al.,* 2017; Chia-chung Hou *et al.,* 2007; Chiatanya *et al.,* 2013; Tomimori *et al.,* 2012).

- Development of affordable drug/therapy can greatly alleviate challenges faced in treatment of blood coagulation challenges in diabetes. Thus, *C. crepidioides* could be a potential herb for this purpose.

Objective of the study

To investigate the effects of *C. crepidioides* extract and fractions on blood coagulation profile of diabetic male Wistar rats.

Specific objectives are to:

- determine the effects of leaf methanol extract and fractions of *C. crepidioides* on blood clotting time and bleeding time in diabetic rats.
- test the effects of leaf methanol extract and fractions of *C. crepidioides* on the Prothrombin time (PT) and activated partial thromboplastin time (aPTT) in diabetic rats.
- test the effects of C. crepidioides leaf fractions on plasma calcium concentration Haematological indices in diabetic rats

Assays of the coagulation pathways

Clotting Time (CT)

- Intrinsic pathway

Bleeding Time (BT)

- Platelets Activation and Aggregation

Prothrombin Time (PT)

- Extrinsic pathway

Activated partial thromboplastin time (aPTT)

- Intrinsic pathway (contact activation)
- Common pathway

Black &Selby (2013)

METHODOLOGY

Collection and Identification of Plant materials

C. crepidioides was locally obtained from farms in Ilisan-remo, Ogun State, South Western Nigeria. The plant sample was identified at the IFE herbarium, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. A voucher specimen was deposited with the voucher specimen registration No: **IFE 17634.**

Ethical Approval

Ethical approval was obtained from Babcock University Health Research Ethics Committee (BUHREC) with the Approval No. BU/BUHREC436/17.

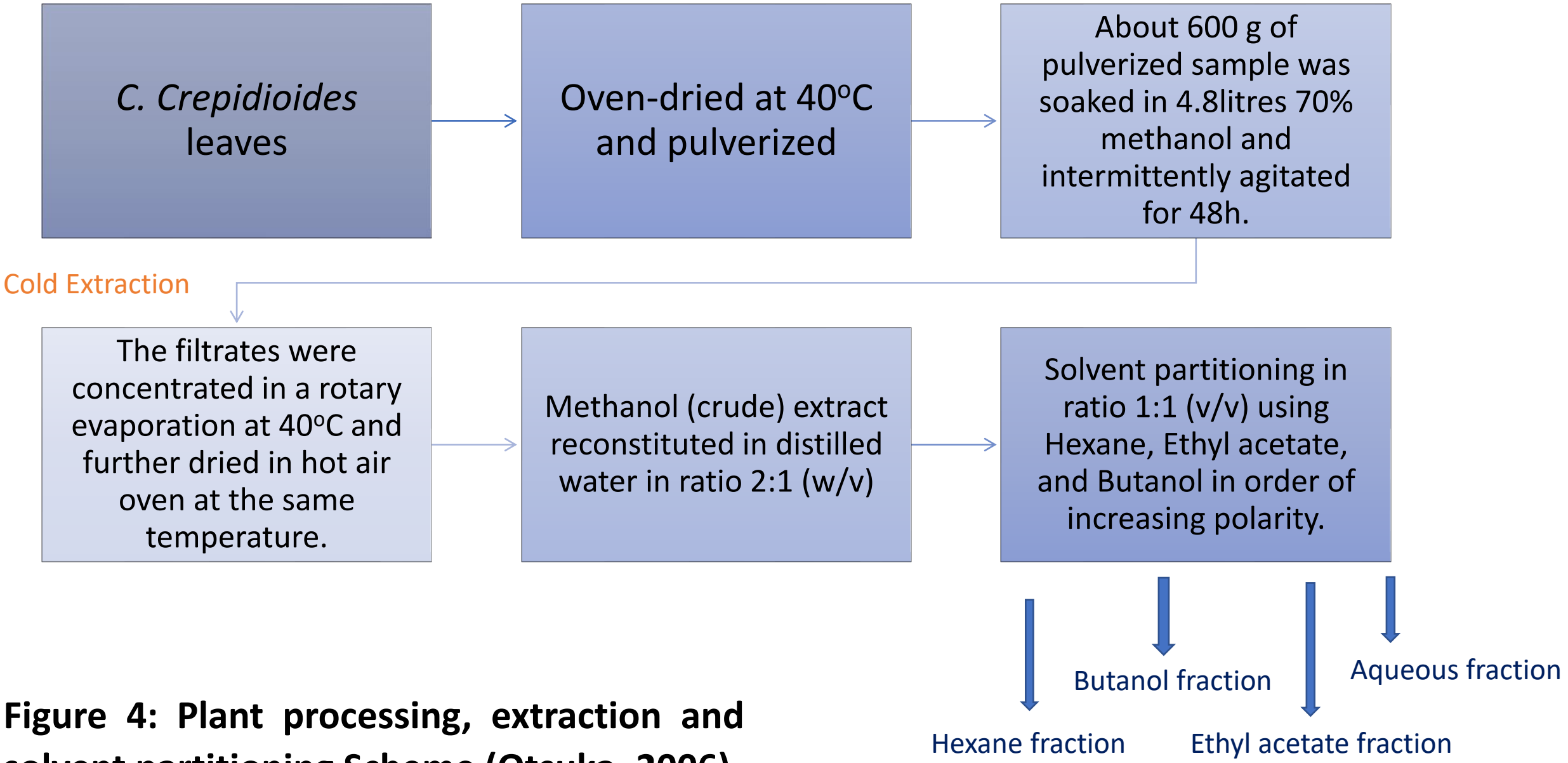

Figure 4: Plant processing, extraction and solvent partitioning Scheme (Otsuka, 2006)

Study Design

In vivo study

Acute Toxicity study with Female Albino Wistar rats (Lorke's Method as reported by Elufioye & Onoja, (2015).

Initial study with the methanol extract and all fractions at 100mg/kg body weight

Main study

66 male Albino rats (150-200g)

Diabetes was induced by single intraperitoneal injection of STZ (55mg/kg body weight) in citrate buffer (pH 4.5).

Fasting blood glucose was checked after 72hrs.

Rats with sustained FBG levels >200 mg/dl were regarded hyperglycemic and used for the experiment.

50-200mg/kg body weight of Aqueous and Hexane fractions were administered orally, once daily using gastric tube for 2 weeks.

Assays

Clotting time and Bleeding time. PT and aPTT.

Calcium concentrations and hematological profile

Experimental Protocol

Group	Description
1	Normal control (given 1ml phosphate buffered saline (PBS).
2	Normal rats given Aspirin dissolved in PBS (75mg/kg body weight) as standard anticoagulant (Nyansah *et al.*, 2016).
3	Diabetic control (given 1ml PBS)
4	Diabetic rats given Aspirin dissolved in PBS (75mg/kg body weight) Nyansah *et al.*, 2016.
5-7	Diabetic rats given the Hexane fraction of *C. crepidioides* suspended in PBS (50, 100 & 200mg/kg respectively).
8-10	Diabetic rats given the Aqueous fraction of *C. crepidioides* suspended in PBS (50, 100 & 200mg/kg respectively).
11	Diabetic rats given Metformin 100mg/kg body weight (Rajesh *et al.*, 2016).

Assay Methods

Methods

- **Clotting time:** Ivy's method as reported by Ibu and Adeniyi (1989).
- **Bleeding time:** method of Shrivasta and Das (1987) as reported by Raaof *et al.,* 2013.
- **PT:** following the PT reagent manufacturer's instruction according to the method of Brown (1988).
- **aPTT:** following the aPTT reagent (Diagen Kaolin Platelet Substitute Mixture) manufacturer's instruction.
- **Hematological profile**: Swelab automatic Autocounter.
- **Plasma calcium:** Randox assay kit.

Statistical Analysis

Data were statistically analyzed by one-way Analysis of Variance (ANOVA) followed by Tukey's Multiple comparisons using Graph pad prism 7.0.

Results were expressed as a Mean ± standard error of mean (SEM). P values less than 0.05 ($p<0.05$) were considered statistically significant.

RESULTS AND KEY FINDINGS

Acute Toxicity

C. Crepidioides failed to produce any adverse effect (after 24hrs) in rats in doses up to 5000mg/kg given orally.
The rats were further observed for 14 days and no mortality or abnormal behaviour was recorded in any of the treatment groups.

Suggested LD_{50} ≥5000 mg/kg.

Coagulation Profile of Experimental rats

Table 1: Coagulation Profile of Diabetic rats treated with methanol extract and fractions of *C. crepidioides* at 100mg/kg body weight

Parameters / Group	Bleeding Time (minutes)	Clotting Time (minutes)	PT (seconds)	aPTT (seconds)
Normal Control	2.00 ± 0.11^{b}	1.58 ± 0.14^{d}	25.00 ± 2.43^{h}	31.00 ± 2.92^{b}
Diabetic control	1.37 ± 0.12^{a}	1.48 ± 0.12^{d}	17.00 ± 2.42^{g}	22.00 ± 0.56^{a}
Hexane	2.39 ± 0.15^{b}	3.45 ± 0.15^{f}	92.00 ± 8.09^{k}	136.00 ± 9.39^{d}
Butanol	2.17 ± 0.16^{b}	2.44 ± 0.10^{e}	81.00 ± 3.63^{k}	74.00 ± 9.32^{c}
Aqueous	4.11 ± 0.50^{c}	3.48 ± 0.19^{f}	66.00 ± 6.37^{j}	126.00 ± 6.96^{d}
Ethyl acetate	3.58 ± 0.40^{c}	2.54 ± 0.13^{e}	74.00 ± 5.53^{k}	74.00 ± 6.91^{c}
Methanol	2.29 ± 0.05^{b}	2.08 ± 0.16^{d}	47.00 ± 2.46^{i}	68.00 ± 1.73^{c}
Metformin	3.50 ± 0.22^{c}	2.58 ± 0.18^{e}	68.00 ± 8.77^{j}	115.00 ± 10.39^{d}

Results are the mean ± SE values of duplicate determinations (n=4).

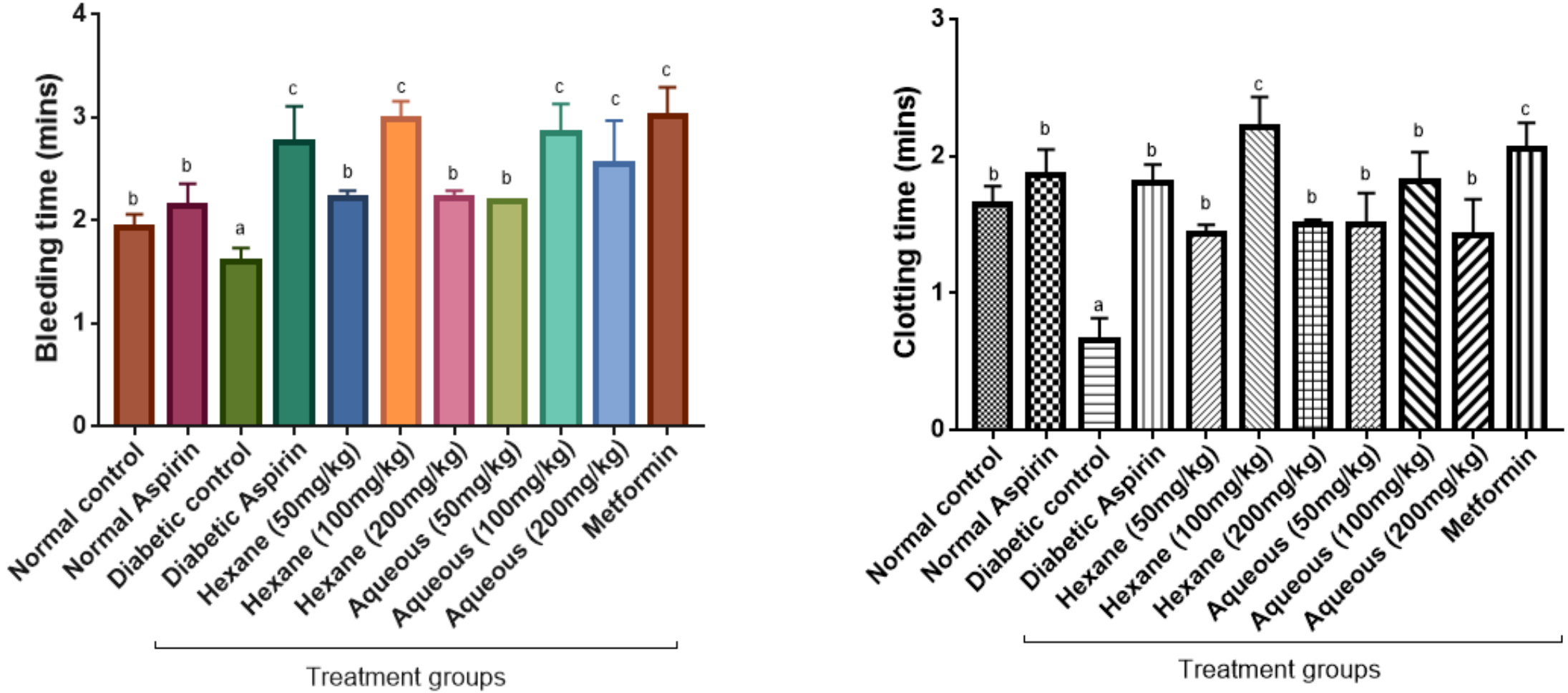

Figure 2: Bleeding Time (left) and Clotting time (right) in Diabetic rats treated with Aqueous and Hexane fractions at different concentrations.
Bars with different letters are significantly different (n=4).

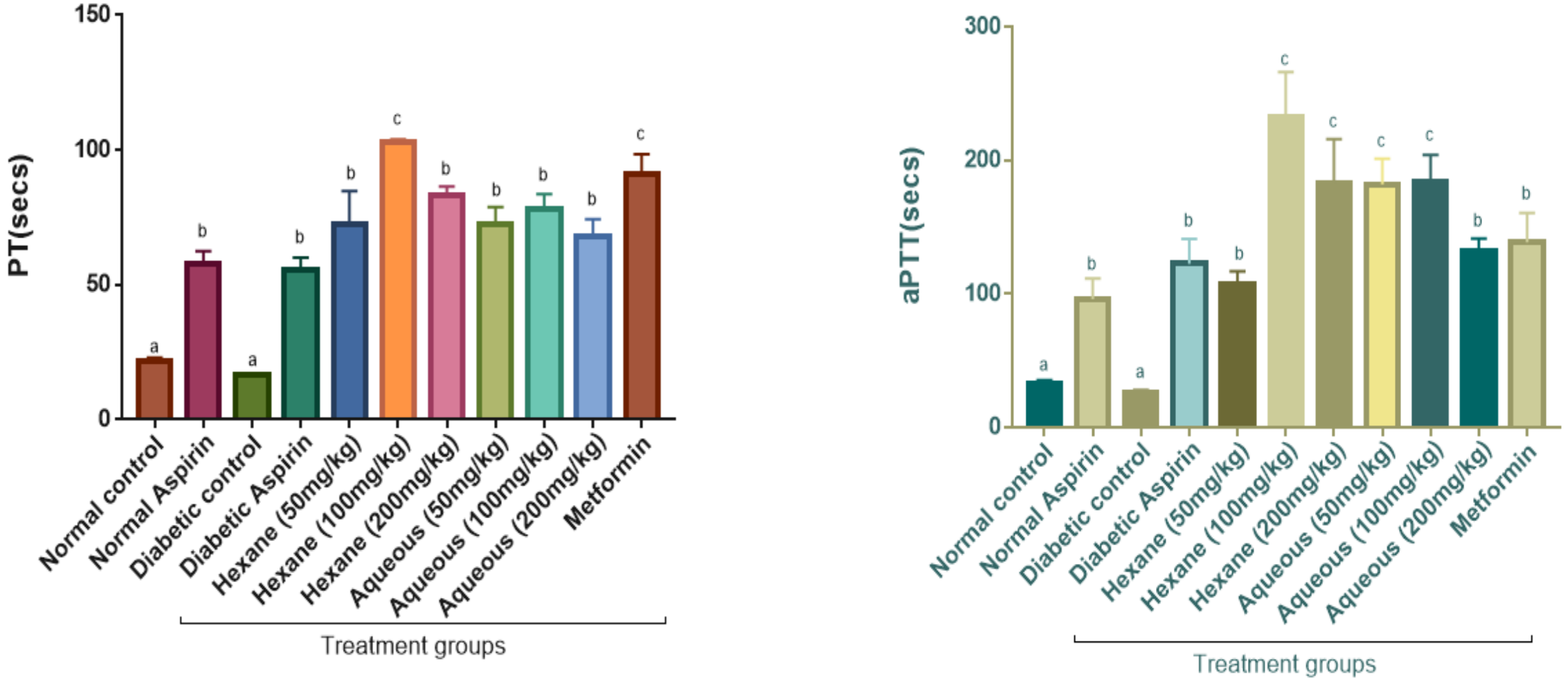

Figure 3: PT (left) and aPTT (right) in Diabetic rats treated with Aqueous and Hexane fractions at different concentrations.

Bars with different letters are significantly different (n=4).

Table 2: Plasma Calcium concentrations of Normal control and Diabetic rats

Parameters Group	Calcium (mg/dl)
Normal Control	8.90 ± 0.03[b]
Normal Aspirin	8.40 ± 0.03[a]
Diabetic control	8.70 ± 0.10[b]
Diabetic Aspirin	8.30 ± 0.03[a]
Hexane (50mg/kg)	8.40 ± 0.03[a]
Hexane (100mg/kg)	8.90 ± 0.03[b]
Hexane (200mg/kg)	8.60 ± 0.03[b]
Aqueous (50mg/kg)	8.50 ± 0.01[a]
Aqueous (100mg/kg)	8.40 ± 0.03[a]
Aqueous (200mg/kg)	8.40 ± 0.03[a]
Metformin	8.50 ± 0.02[a]

Results are the mean ± SE values of duplicate determinations (n=4).

Key findings and Implication(s)

- Shorter bleeding time, PT and aPTT recorded in diabetic control rats compared to Normal control rats indicate hypercoagulation in diabetes.

- All concentrations of *C. Crepidioides* leaf methanol extract and fractions administered to diabetic rats significantly increased all the tested coagulation parameters.

- Highest increase was recorded in diabetic rats treated with 100 mg/kg Hexane fraction.

- The results suggest anticoagulant activity of *C. crepidioides.*

Summary from blood Coagulation results

- In clinical evaluation, abnormalities in both PT and aPTT points at factors V, X and prothrombin (factor II) of the common pathway (Jesty, 2013).

- Therefore, the prolonged PT and aPTT after *C. crepidioides* administration observed in the study suggests inhibition of factors V, X and prothrombin by *C. crepidioides*.

- *C. crepidioides* active component may also lower intracellular calcium thus limiting calcium available for the formation of Tenase (IXa:VIIIa) and prothrombinase (Xa:Va) complex necessary for activation of prothrombin to thrombin.

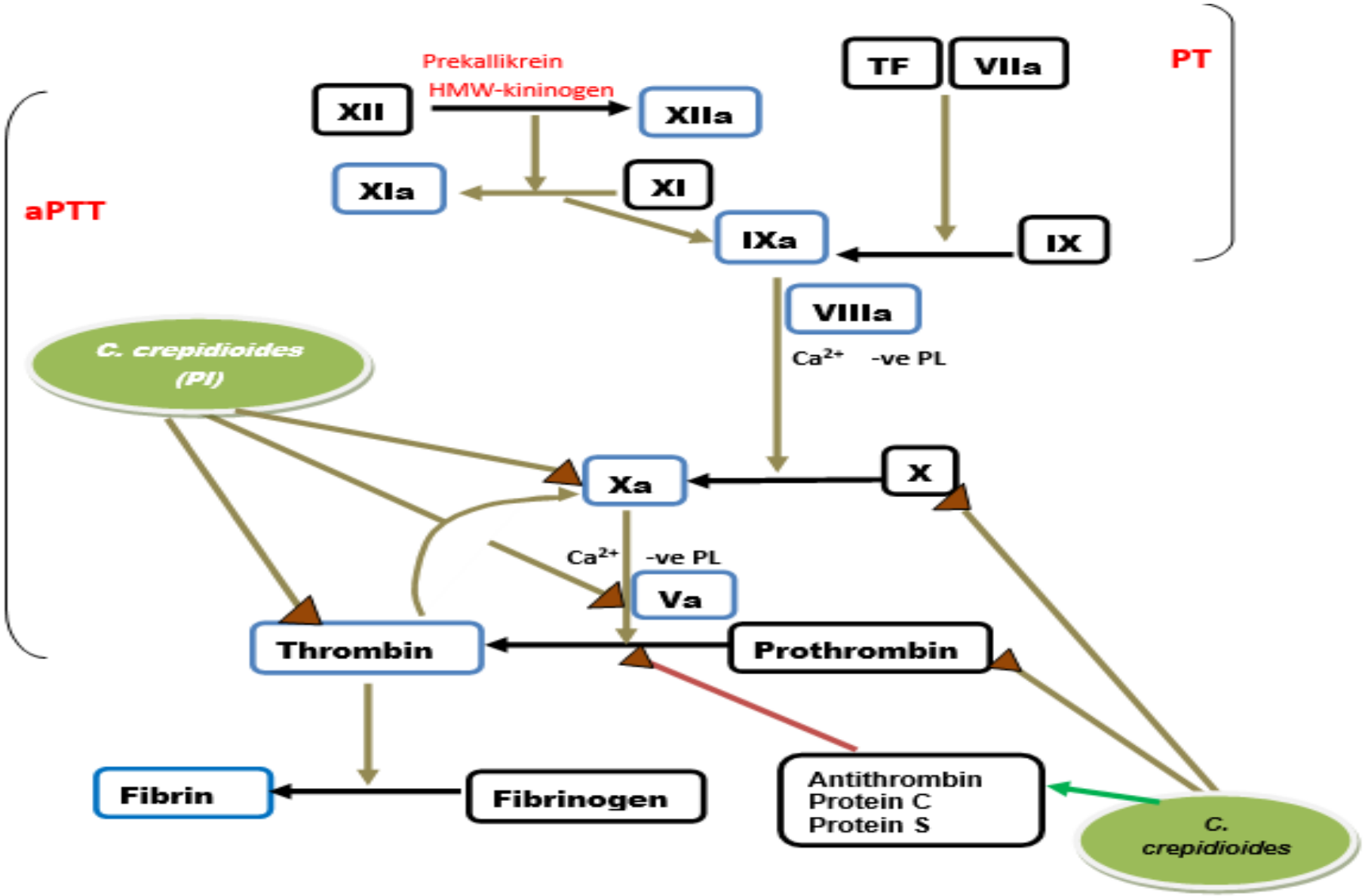

Figure 4 : Proposed model for mechanism of anticoagulant activity of *C. crepidioides*

Table 3: Effect of *C. crepidioides* extract and fractions on Haematological profile of diabetic rats

Parameters	Normal control	Normal Aspirin	Diab control	Diab Aspirin	Hex 50mg/kg	Hex 100mg/kg	Hex 200mg/kg	Aq. 50mg/kg	Aq. 100mg/kg	Aq. 200mg/kg	Metformin
RBC (x 10^{12}/L)	7.70 ±0.01^{b}	7.01 ±0.66^{b}	6.44 ±0.10^{a}	6.86 ±0.19^{b}	7.13 ±0.12^{b}	7.36 ±0.14^{b}	6.50 ±0.40^{a}	6.81 ±0.08^{b}	7.22 ±0.37^{b}	6.45 ±0.03^{a}	7.30 ±0.13^{b}
PCV (%)	43.17 ±0.49^{h}	39.53 ±2.22^{g}	32.10 ±1.13^{f}	38.60 ±1.33^{g}	33.23 ±6.68^{f}	42.20 ±0.23^{h}	38.13 ±0.78^{g}	36.90 ±0.72^{g}	39.50 ±1.16^{g}	34.90 ±0.64^{g}	38.73 ±0.09^{g}
HGB (g/dl)	14.13 ±0.09^{b}	13.57 ±0.67^{b}	11.20 ±0.31^{c}	11.40 ±0.40^{c}	13.40 ±0.23^{b}	13.73 ±0.07^{b}	12.63 ±0.23^{b}	12.40 ±0.29^{b}	14.20 ±0.69^{b}	12.53 ±0.03^{b}	13.27 ±0.19^{b}
PLT (x10^{9}/L)	518.00 ±6.25^{m}	516.33 ±1.16^{m}	485.67 ±22.2^{m}	333.33 ±18.49^{l}	345.67 ±18.41^{l}	207.33 ±4.37^{k}	337.67 ±12.02^{l}	264.33 ±26.57^{k}	388.33 ±18.95^{l}	265.67 ±25.53^{k}	383.67 ±3.84^{l}

Table 4: Some GC – MS Identified Phytochemical components of the Hexane fraction of *C. crepidioides* leaf extract

S/N	Retention time (mins)	Name of compound (Library ID)	Molecular formula	Peak Area (%)	Reported Biological Activity (Duke 2013, 2016)
1	3.586	Butyrolactone	$C_4H_6O_2$	0.98	Antimicrobial. Central nervous system depressant (CNS) and hypnotic. Anaesthetic.
2	5.449	Benzene acetaldehyde	C_8H_8O	1.11	Antioxidant Antibacterial, Anaesthetic.
3	10.286	Benzofuran	C_8H_6O	1.43	Antidepressant, Anticancer, antiviral, antifungal, antioxidant, anti-psychotic, anti-inflammatory.
4	19.795	Benzofuranone	C_8H_6O	2.99	Antioxidant, Anticancer
5	13.640	Thujone	$C_{10}H_{16}O$	0.56	Antibacterial, Antifungal, Antinociceptive, Insecticidal, Anthelmintic Antioxidant (Duke, 2013), Antiplatelet (Cordier & Steekamp, 2011).
6	14.180	Eugenol	$C_{10}H_{12}O_2$	4.43	Anti-inflammatory, Antiseptic (Bandre *et al.*, 2016), Anticoagulant, Antiaggregant (Kim *et al.*, 2010).
7	22.151	1,9 octadecadiene	$C_{18}H_{34}$	0.78	Not stated
8	27.250	n-Hexadecanoic acid	C16H32O2	1.19	Antioxidant, anti-inflammation Hypocholesterolemic, Nematicide Pesticide, Lubricant, Antiandrogenic, 5-alpha reductase inhibitor.
9	29.404	9,12,15-Octadecatrienoic acid (a-linolenic acid)	$C_{18}H_{30}O_2$	4.52	Anti-Inflammatory, Hypolipidemic, Antiaggregant, Anti-leukotriene, Antiprostatic, Immunostimulant, Vasodilator, 5-alpha reductase inhibitor

CONCLUSION

The study has shown that *C. crepidioides* possesses anticoagulant and anti-anaemic activities.

The leaves can thus be a potential source of novel anticoagulant and nutraceutical for management of thrombotic disorder in diabetes and other diseased states.

Further toxicological study is required to ensure the plant safety on internal organs of the body.

For listening