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Total Phenolic, Flavonoid and Carotinoid Content and Anticoagulation Activities of Sapparin Tablet

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Authors' contributions

This work was carried out in collaboration among all authors. Author EC carried out the phytochemical study and wrote the section of the phytochemistry in the manuscript. Author D. Buyantogtokh determined data analysis and wrote the section of the pharmacology in the manuscript. Author D. Begzsuren advised the work. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Sapparin tablet, a medicine used for the treatment of blood diseases specially curing blood thickening or impure blood, liver disease in Mongolian Traditional Medicine. The objectives of the study were to determine total biological active substances and analyze the anticoagulation activity of the Sapparin.
Study Design: Experimental study.
Place and Duration of Study: Department of Chemistry and Technology and Department of Pharmacology, Institute of Traditional Medicine and Technology of Mongolia.
Methodology: Quantitative determination of the total active constituents (phenolic, flavonoid, and carotinoids) of the methanol extracts of Sapparin was performed by using Folin-Ciocalteu reagent, aluminium chloride reagent by spectrophotometry.
A totally of forty weighing between 220-250 gm were used. Effect of Sapparin was assessed on

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coagulation parameters following 7, 14, 21 and 28 days administration of 37 mg/kg, 56 mg/kg, 113 mg/kg to healthy rats. The blood coagulation parameters such as prothrombin time (PT) and the activated partial thromboplastin time (aPTT) were measured by means of Quick's one-stage assay and modified aPTT assay respectively in the rats. Additionally, thrombin activity test was estimated in rats with PT assay using a hemagglutination analyzer. The levels of serum X and von Willebrand factor were measured in Sapparin and control groups by ELISA.

Results: The total content of the phenols measured as 5.33±0.0005%, flavonoids as 12.95± 2.21% and carotinoids as 4.31±0.96%.

There was significant increase in all assays except fibrinogen, prothrombin time, thrombin, aPTT. Sapparin treatment significantly reduced levels of serum X factor and von Willebrand factor was significantly decreased in rats.

Conclusion: Results of this study suggest that Sapparin shows considerable anti-anticoagulant activity in animals and has potential to reduce cardiovascular morbidity and mortality.

Keywords: Herbal medicine; sapparin; thrombin time; prothrombin time; activated partial thromboplastin time; total phenolic; flavonoids; carotinoids.

1. INTRODUCTION

According to the theory of traditional Mongolian medicine, blood essences disturbed by the condition of disease development, and hence blood disease arises. On the other hand, symptoms of blood thickening and forming thrombosis are considered as a disease occurring by means of an impure blood [1]. In the Mongolian medicine "Sapparin" tablet is used for curing blood thickening or impure blood. Thus, we chose to study the anticoagulation activity of the Sapparin tablet in laboratory animals. Sapparin tablet is a composed of 4 medicinal plants including *Caragana jubata (*Pall.) Poiret.*, Caesealpinia sappan* L.*, Zingiber officinalie* Willd., *Hippophae rhamnoides* L. [1,2] .

Caragana jubata (Pall.) Poir. is a perennial leguminous bush endemic in the Northwest of Mongolia and China. It is one of the oldest medicinal plants used in traditional Tibetan medicines. The whole plants of *C. jubata* have been long used to treat some cardiovascular diseases, such as atherosclerosis, hyperlipidemia, hypertension, blood circulation disorder, blood stasis and etcn [3]. The species contained flavonoids and stilbenoids, where flavonoids constituted majority of compounds isolated from it in the late 20th century. Chemical components, including flavonoids (myricetin, quercetin, kaempferol) and O-methylated flavonols (isorhamnetin, laricitrin, syringetin), resveratrol, cassigarol E, scirpusin B, a few volatile oils and four pterocarpan glycosides were isolated from *Caragana jubata (*Pall.) Poiret [4,5].

Caesealpinia sappan L. is widely used as a Chinese and traditional Mongolian medicine for the treatment of menorrhalgia, urogenital, cardiovascular and cerebrovascular blood diseases. Phytochemical constituents of Sappan wood have been studied resulting in the separation of various components including homoisoflavonoids, diterpenoids, dibenzoxocins, and a lactone [6]. Chemical constituent's investigation of Sappan wood resulted in the isolation of various structural types of phenolic components including xanthone, coumarin, chalcones, many flavones, homoisoflavonoids and brazilin. Brazilin is a biologically active substance and active compound found in *Caesealpinia sappan* L. Most of brazilin uses were validated by scientific studies such as antioxidant, antibacterial, anti-inflammatory, anti-photoaging, the inhibitory effects of brazilin on Zn^{2+}-mediated Aβ aggregation, hypoglycemic, vasorelaxant, hepatoprotective and anti-acne activity [7]. This biologically active substance is non-toxic and if safely used, the compound has potential to develop as a medicinal compound with application in cosmetics and pharmaceutical industries.

Phytochemical analysis of *Zingiber officinalie* Willd, showed the presence of active ingredients such as gingerol, shogoal, zingerone, paradol, zingerberene and other terpenoids and flavonoids, which are responsible for its various ethnomedical significance and biological activities [8]. It has been investigated effects on the gastrointestinal tract, cardiovascular system, blood pressure, blood clotting and antimicrobial effects [9].

Hippophae rhamnoides L. have been found to have a high content of many bioactive compounds such vitamin C, carotenoids, tocochromanols, phenolic compounds, folates

and healthy fatty acids. Sea buckthorn has also been shown to have positive effects on cardiovascular disease by inhibiting platelet aggregation and oxidation on low density lipoprotein [10].

Venous thromboembolism (VTE) is a disorder that includes deep vein thrombosis and pulmonary embolism. A deep vein thrombosis (DVT) occurs when a blood clot forms in a deep vein, usually in the lower leg, thigh, or pelvis. A pulmonary embolism (PE) occurs when a clot breaks loose and travels through the bloodstream to the lungs. Deep vein thrombosis and pulmonary embolism is a major cause of morbidity and mortality in the world. It is also a serious health care problem in the world, which plays an important role in the pathogenesis and progression of atherosclerosis, cardiovascular diseases and diabetic complications [11]. Nowadays the risk factors for thrombosis include blood stasis, vessel wall injury, and hypercoagulability, as proposed by Virchow over 150 years ago [12,13].

Therefore, it was postulated that Sapparin tablet could be an anticoagulant. In the present study, this hypothesis was tested using a measured prothrombin time (PT), thrombin time (TT), the activated partial thromboplastin time (aPTT), levels of serum X and von Willebrand factor in rats.

2. MATERIALS AND METHODS

2.1 Plant Materials

The crude herbal medicines *Caesealpinia sappan* L.*, Zingiber officinalie* Willd, were purchased from a Traditional drug factory at the Institute of Traditional medicine and Technology (Ulaanbaatar, Mongolia). *Caragana jubata (*Pall.) Poiret. herbs were collected from Arkhangai, Mongolia in 2018 and *Hippophae rhamnoides* L. was cultivated in Bulgan, Mongolia. The origin of each herbal medicine was taxonomically confirmed by Prof. Ganbold E, Ulaanbaatar University, Ulaanbaatar, Mongolia. The herbarium specimens for these plants deposited in Institute of Traditional Medicine and Technology, Mongolia.

2.2 Experimental Animals

A totally of forty healthy male Wistar rat weighing between 220-250 gm were purchased from the Experimental Animal Center, Institute of Traditional Medicine and Technology of Mongolia. They were kept under controlled conditions of temperature (20±1c°) and humidity (about 50-60%), with a 12-hour light/dark cycle, and automatic ventilation 8-15 times every hour. Rats could drink ad libitum, and were fed with standard nutrient.

2.4 Reagent

Standards of gallic acid, rutin, β-carotene, brazilin, quercetin and keampferol were obtained from Sigma-Aldrich (USA). The Folin Ciocalteu's phenol reagent and aluminium chloride (AlCl3) of Sangon, China used in the study. All other solvents and chemicals were of analytical grade.

2.5 Chemical Analysis

2.5.1 Sample preparation

A powdered medicine was precisely weighed (1.0 g), and extracted with 50 ml of 70% ethanol in reflux for 30 min, and filtrated. The supernatant was used as a test solution.

2.5.2 Thin layer chromatography

Thin layer chromatographic (TLC) plates, composed of Merck Silica gel 60 GF 254, received 5 µL of the test solutions placed at a distance of 1.5 cm of the lower edge of the plate. The mobile phase was toluene / ethylacetate / formic acid (60:40:3, v/v) for flavonoids and spray with aliminium chloride [14], ethylacetate / toluene / acetic acid (7:3:1, v/v) for brazilin [15] and hexane / acetone (3:2, v/v) for β-carotene and dry in air [16].

2.5.3 Estimation of total flavonoid contents

The solution was treated with 1 ml of the 5% NaNO2, 1 ml of the 10% Al(NO3)3 and 10 ml of the 4% NaOH solution was added, and value of absorbance was determined using spectrophotometer (UNICO UV-2102 C, China) at 500 nm. The content of flavonoids in extracts was expressed as rutin equivalent (mg of RU/g of extract) [17].

2.5.4 Estimation of total polyphenolic compounds

The amount of total phenolics was determined using Folin-Ciocalteu assay. The Folin-Ciocalteu reagent (diluted 1:10 in water) and aqueous Na2CO3 (10.75%) were successively added to the extract. In 30 min value of absorbance was

measured at 760 nm. Gallic acid was used to establish the calibration curve, and total polyphenolic content was expressed as g/kg [18].

2.5.5 Estimation of total carotinoids

20ml of Petroleum ether were pipetted into a separating funnel with Teflon stopcock. 15 ml of the acetone extract were added and allowed to stand for 15 minutes. 150 ml of distilled water were added by flowing along the walls of the funnel. The mixture was allowed to separate into two phases, and the aqueous phase was discarded. The petroleum ether phase was washed 4 times with 100 ml of distilled water to remove residual acetone. The petroleum ether phase was collected in a 25 ml volumetric flask by passing the solution through a small funnel containing 7.5 g of anhydrous sodium sulfate to remove residual water. The separating funnel was then washed with petroleum ether and the washing was collected into the volumetric flask by passing it through the funnel with sodium sulfate. The volumetric flask was then made up to volume with petroleum ether and the total carotenoids content were determined from the molar absorptivity β-carotene E1% = 2590 at λmax 450nm derived from the standard plots [19].

2.6 Anticoagulation Activities in Sapparin Tablet

2.6.1 Experimental protocol

Forty rats randomly divided into four groups of ten rats each (30 Sapparin treated, 10 normal rats) used for the study. Group 1: normal control rats received distilled water daily, orally by gavages, 7, 14, 21, 28 days, Group 2: received Sapparin 37 mg/kg, Group 3: Sapparin 56 mg/kg, Group 4: Sapparin 116 mg/kg daily for a 4 week.

2.6.2 Determination of prothrombin time, activated partial thromboplastin time and thrombin activity in rats

PT, aPTT and thrombin activity were estimated by reported standard methods as indication for blood coagulation. PT was measured by means of Quick's one-stage prothrombin test and aPTT by means of modified aPTT assay using an EA-containing aPTT reagent. The thrombin activity was determined by PT assay with a PK-B hemagglutination analyzer (Zhongshan Peikang Limited Company for Medical Electronic Instruments, Zhongshan, China).

2.6.3 Plasma levels of cytokines (X factor and von Willebrand factor)

A 4-5 ml blood sample was collected from each rat by cardiac puncture. The serum was separated by centrifugation at 3000 rpm for 15 minutes. The level of plasma X factor and von Willebrand factor were measured by ELISA following the kit's instructions (Chromate 4300 microplate, Shanghai MLBIO Biotechnology Co. Ltd., China).

2.7 Statistical Analysis

Data was reported as means±SD. Statistical significance was determined by one-way analysis of variance followed by Tukey's multiple comparison test. A P-value of 0.05 was considered statistically significant.

3. RESULTS

3.1 Thin layer chromatography

TLC fingerprints of reference standards and various Sapparin extracts are showed in Figs. 1-3. All extracts presented chromatographic bands corresponding to that of standard β-carotene, brazilin, quercetin and keampferol. Rf value were 0.39, 0.55, 0.71 and 0.95 for quercetin and keampferol, brazilin and β-carotene, respectively.

3.2 Total Phenolic, Flavonoid and Carotinoid Contents

The flavonoid contents of the extract in terms of rutin equivalent (the stander curve equation: y =11.815x – 0.0092 r2 = 1.000) were between 4.0 to 40.0 (Table1). The flavonoid content in the extract of Sapparin tablet (12.95± 2.21%). Table 1 also shows the contents of total phenols that were measured by Folin Ciocalteu reagent expressed as gallic acid equivalent (the stander curve equation: y =110.77 x - 0.0736, r2 = 0.995) were between 0.72 to 2.1μg/ml. The total phenol varied from 5.33±0.0005% in the Sapparin. The content of carotinoids were measured by spectrophotometer in term of β-carotene equivalent contents and was found as 4.31±0.96% in Sapparin tablet (Table1).

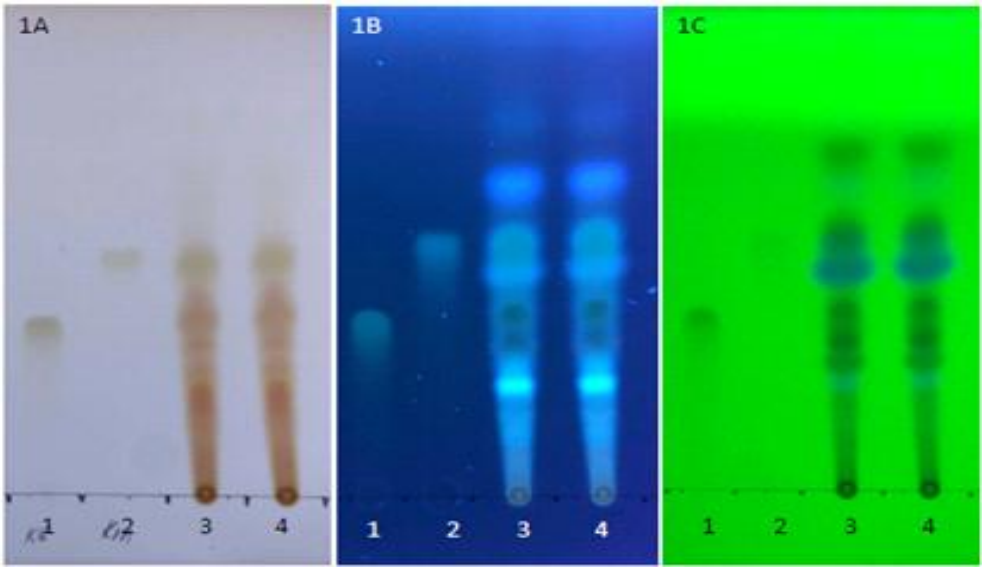

Fig. 1. TLC fingerprint of 1.Standard quercetin, 2.Standard keampferol, 3-4.Extract of Sapparin tablet; *A. Spray* with aliminium chloride, B.UV 365 nm, C.UV 254nm;

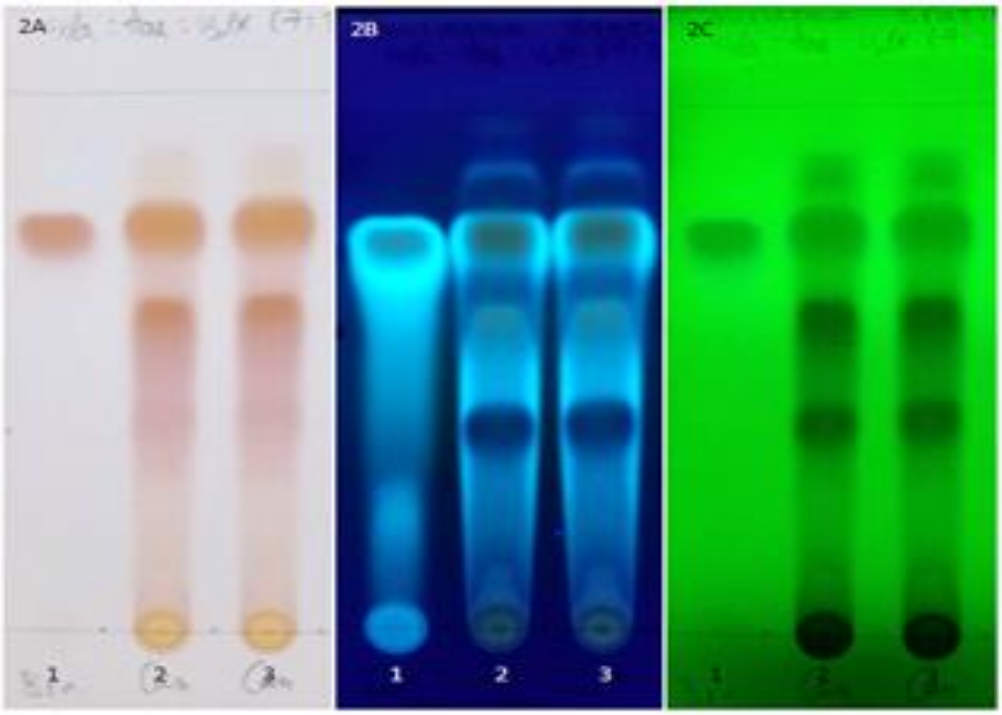

Fig. 2. TLC fingerprint of 1.Standard brazilin, 2-3.Extract of Sapparin tablet; A. Natural light, B.UV 365 nm, C.UV 254nm;

Table 1. Total phenolics, flavonoids and carotinoids in methanol extracts of the Sapparin tablet (n=6, % dry mass)

Bioactive substance	Values obtained
Flavonoids, %	12.95± 2.21
Total phenolics, %	5.33±0.0005
Total carotinoids, %	4.31±0.96

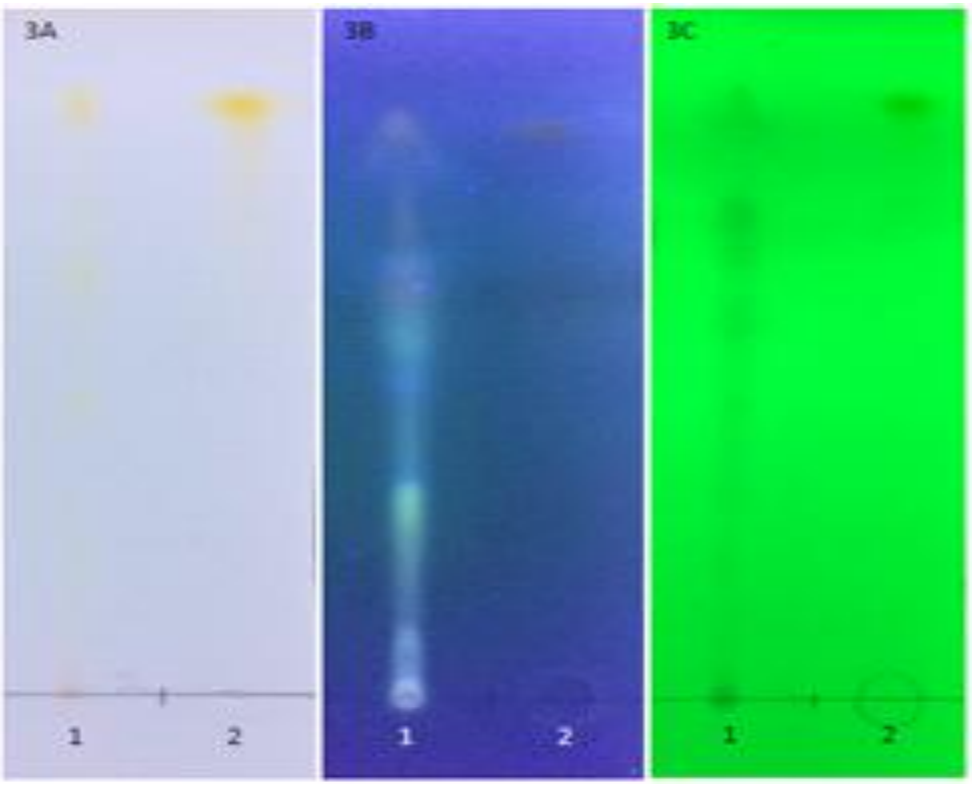

Fig. 3. TLC fingerprint of 1. Standard β-carotene, 2. Extract of Sapparin tablet; A. Natural light, B.UV 365 nm, C.UV 254nm

3.3 Effects of Sapparin on Anticoagulation Activity in Rats

There was significant shortened in TT at 37 mg/kg and 113 mg/kg doses. There was significantly prolonged in aPTT at three doses and highly significantly prolonged was observed with moderate and high dose of Sapparin. PT was not affected significantly at any dose. Fibrinogen was decreased in 56 mg/kg dose.

Table 2. Anticoagulation effect of Sapparin on coagulation parameters at 7 days

Parameters	Experimental animal groups			
		Sapparin mg/kg		
	Control	37	56	113
PT (seconds)	10.1±0.7	9.3±0.3	9.5±0.25	10.6±0.3
TT (seconds)	44.0±4.2	31.5±2.1*	42.3±3.1	34.5±1.4*
aPTT (seconds)	13.9±0.7	17.1±1.1**	16.4±1.4*	16.02±1.0*
Fib (g/L)	2.3±0.2	2.4±0.2	1.9±0.09*	2.4±0.4

*n=10, mean±SD, *P< .05, **P< .001 as compared to control*

Table 3. Anticoagulation effect of Sapparin on coagulation parameters at 14 days

Parameters	Experimental animal groups			
		Sapparin mg/kg		
	Control	37	56	113
PT (seconds)	10.1±0.7	12.7±1.1*	10.5±1.2	11.1±0.5
TT (seconds)	44.0±4.2	47.2±4.6	51.7±4.5*	63.1±3.5**
aPTT (seconds)	13.9±0.7	20.1±1.9***	17.7±0.8**	14.4±0.7
Fib (g/L)	2.3±0.2	2.3±0.1	0.8±0.05**	1.9±0.4*

*n=10, mean±SD, *P< .05, **P< .001 as compared to control*

Table 3 shows the effect of Sapparin on coagulation parameters at 14 days. There was highly significant prolonged in TT 56, 113 mg/kg doses and significant prolonged in PT at 37 mg/kg doses. aPTT was significantly prolonged at 37, 56mg/kg doses. Fibrinogen amount was decreased significantly at 56, 113 mg/kg doses.

Table 4 shows that significant prolonged in PT at 56, 113 mg/kg, at 21 days, aPPT was significantly prolonged moderate dose, but fibrinogen was decreased high significantly all doses. TT had no significant changes at any dose.

While Table 5 illustrated that there was a significant fall in Fibrinogen at all doses, at 28 days. aPPT was prolonged at doses.

Fig. 4A experienced that there was a significant decrease in X factor at all dose, while Fig, 4B showed to significantly decreased in VWF at 37, 56 mg/kg doses, after 21 days.

Table 4. Anticoagulation effect of Sapparin on coagulation parameters at 21 days

Parameters	Experimental animal groups			
		Sapparin mg/kg		
	Control	37	56	113
PT (seconds)	10.1±0.7	10.8±0.3	12.4±1.6*	14.3±1.6*
TT (seconds)	44.0±4.2	44.0±3.7	40.2±1.3	37.7±3.9
aPTT (seconds)	13.9±0.7	13.0±1.3	17.1±0.3**	14.3±0.6
Fib (g/L)	2.3±0.2	0.6±0.09***	0.8±0.1**	0.7±0.1**

*n=10, mean±SD, *P< .05, **P< .001, ***P< .0009 as compared to control*

Table 5. Anticoagulation effect of Sapparin on coagulation parameters at 28 days

Parameters	Experimental animal groups			
		Sapparin mg/kg		
	Control	37	56	113
PT (seconds)	10.1±0.7	10.2±0.7	13.0±.08*	10.02±0.5
TT (seconds)	44.0±4.2	45.5±4.5	48.2±2.1	51.5±5.7*
aPTT (seconds)	13.9±0.7	20.0±1.8***	19.1±1.4***	16.5±1.2*
Fib (g/L)	2.3±0.2	1.6±0.04*	1.7±0.3*	1.1±0.1**

*n=10, mean±SD, *P< .05, **P< .001, ***P< .0001 as compared to control*

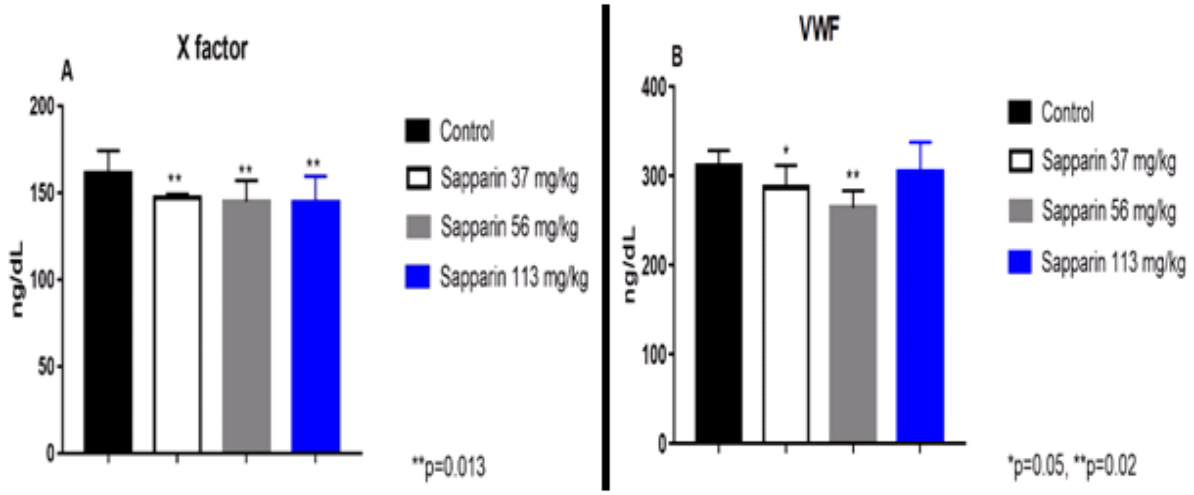

Fig. 4. Changes in the levels of clotting factors a, x factor; b, von-willebrand (vwf)
Each group: n=10 for Data are reported as means±SD, (ANOVA)

4. DISCUSSION

Components contained in Sapparin tablet were identified using thin layer chromatography (TLC) with β-carotene, brazilin, quercetin and keampferol as standard. TLC result illustrated Sapparin tablet contained same Rf and bands color as β-carotene, brazilin, quercetin and kaempferol. In TLC fingerprint that identified brazilin, there were 2 red-brown bands on visible light, muffled color in UV 254 and green fluorescence on UV 366. This showed probability of Sapparin contains brazilin and similarly to compare the result of study of Asri Mega Putri et al. [15]. However, standardized method of extraction and TLC is needed to get more validated result.

Flavonoids are the main compound in Sapparin, content was determined 12.95±2.21%, and flavonoids are a group of polyphenolic compounds and exhibit several biological effects such as antihepatotoxic, anti-inflammatory, anti-ulcer activity and anticoagulation activities. For example, Guglielmone et al found that 3-acetyl-7,3',4'-trisulphate (ATS) and quercetin-3,7,3',4'-tetrasulphate (QTS) obtained from Flaveria bidentis showed significant prolongation of the activated partial thromboplastin time (aPTT), less for the prothrombin time (PT), and had no effect on the thrombin time (TT) at a concentration of 1 mM [20].

Ethanolic solution of Rutin at an overall concentration of 830 μM (which corresponds to a mol fraction of 60 μM of anionic Rut form), showed prolongation of aPTT due to interaction with factors VIII and IX. All investigated complexes prolonged only aPTT, (Rut-Al and Hesp-Cu significantly, $p < 0.001$) and had no effects on PT and TT [21].

Many natural substances such as quercetin, keampferol, luteolin have shown the significant prolongation of the activated partial thromboplastin time (aPTT), less for the prothrombin time (PT), due to interaction with factors VIII and IX [22].

We evaluated for the anticoagulant the effect of a tablet of Sapparin in the rats. The result of this coagulation test revealed that anticoagulation effect Sapparin significantly prolonged PT, aPTT, TT and decreased fibrinogen amount. Von Willebrand's factor is a glycoprotein in hemostasis. These results suggested that Sapparin could decreased Von Willebrand's factor and result in anticoagulant than other treatments by regulating Von Willebrand's factor in the coagulation process. In the present study, the levels of X factor were decreased in all groups. Sapparin believes that most stages of the coagulation.

Therefore, Sapparin tablet component's anticoagulant activity was studied separately, which indicates its anticoagulant action once again. For example, *Caesalpinia sappan* L and *Caragana jubata* Pall Poir. are an effective anticoagulant and antithrombotic agent [23].

The Ginger Rhizome Methanolic Extract significantly prolonged PT, aPTT and TT, compared to the control [24] and Ginger (*Zingiber officinale*) has been shown to inhibit platelet aggregation 7, 8, 13 and to decrease platelet thromboxane production *in vitro* [25].

Thus, the aPTT, PT and TT were tested. The results demonstrated that Sapparin prolonged aPTT, PT and TT but showed more a powerful effect on aPPT and TT than PT, suggesting that Sapparin mainly inhibited intrinsic pathway of coagulation and fibrin formation. In addition, Sapparin has decreased activity clotting factors (X, VWF) in rats.

5. CONCLUSION

In conclusion, the mainly compound of Sapparin tablet is flavonoids and it is anticoagulation activity due to the result of coagulation test revealed that Sapparin significantly prolonged PT, aPTT, TT and decreased fibrinogen amount while experienced there are a fall of Von Willebrand's factor.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The study was carried out in accordance with the Health Ethics Guidelines issued by the Mongolian Ministry of Health (2018). The study protocol (№02/02/2018-06) was approved by members of "The Research Ethics Committee" and by the Institute of Traditional medicine and technology.

RESEARCH SIGNIFICANCE

The study highlights the efficacy of "herbal medicine" which is an ancient tradition, used in some parts of Mongolia. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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