Original Research Article

Relaxant Activities of Extracts from *Uvaria rufa* Blume and *Caesalpinia sappan* L. on Excised Rat's Prostate Strips

ABSTRACT

Aims: To determine the relaxant activity of various extracts from the stems of *Uraria rufa* Blume and *Caesalpinia sappan* L. on rats' prostate strips *in vitro*.

Study design: The relaxant efficacies of ethyl acetate and ethanolic extracts from the stems of *U. rufa* and *C. sappan* were tested on isolated rats' prostate tissue pre-contracted by adrenaline. These were compared to tamsulosin, a synthetic drug. Phytochemical constituents including total phenolics and total flavonoids of each extracts were investigated.

Place and Duration of Study: Department of Biology, Faculty of Science, Chiang Mai University, between February and September 2018.

Methodology: The prostate smooth tissue strips were isolated from male Wistar rats and mounted in an organ bath filled with Krebs-Henseleit solution pre-warmed to 37 ^{o} C and continuously aerated with 5 % carbon dioxide in oxygen. To measure a postjunctional effects to the extracts, a prostate strip was induced to contract by adrenaline at 0.001-100 µM and the contracted strip was then exposed to each at 250 µg/mL for 30 minutes. The tension was recorded by a SS12LA force transducer connected to the Biopac Student Lab PRO® 3.7 Software. Relaxant efficacies of various extracts were determined in prostate strips pre-contracted by adrenaline at 10 µM. Percent relaxation, percent maximal effect (E_{max}), and effective concentration of compound to produce 50 % of relaxation (EC_{50}), were determined. All extracts were screened for the presence of bioactive components. The contents of total phenolics and total flavonoids in various extract were measured.

Results: The results showed that all of the extracts, as well as tamsulosin, a synthetic drug, exhibited relaxant effects ($P < 0.001$) on prostate smooth muscles. The ethyl acetate extract of *U. rufa* exhibited the most potency in relaxing the prostate smooth muscle (E_{max} = 72.09 ± 2.15 %). The EC_{50} values of the ethyl acetate extract of *U. rufa*, ethanolic extracts of *C. sappan* and *U. rufa* and ethyl acetate extract of *C. sappan* were 140.23 ± 9.74, 226.35 ± 7.16, 235.35 ± 24.96 and 236.24 ± 5.05 µg/ml respectively, while tamsulosin was 86.83 ± 8.96 µg/ml. Preliminary phytochemical screening showed the presences of flavonoids, phenolics, sterols, tannins, phlobatannins, terpenoids, cardiac glycosides, alkaloids and reducing sugars in all extracts. The highest contents of phenolics and flavonoids were found in the ethanolic and ethyl acetate extract of *C. sappan* respectively.

Conclusion: We concluded that the ethyl acetate from the stems of *U. rufa* was the most potent in relaxing the prostate smooth muscles, and it may be useful to relieve the urological symptoms caused by the BPH.

Comment [U1]: Benign prostatic hyperplasia (BPH)

Keywords: Benign prostatic hyperplasia; Caesalpenia sappan L.; prostate; relaxation; Uvaria rufa Blume

1. INTRODUCTION

Benign prostatic hyperplasia (BPH) is identified by the hyperproliferation of both static and dynamic components leading to nonmalignant prostate enlargement [1]. The growth of a static component or prostatic epithelium is regulated by the more potent androgen, dihydrotestosterone (DHT), which is converted from testosterone by the 5α-reductase enzyme.

The dynamic component or stromal smooth muscle is regulated by the sympathetic nervous system. Clinical studies have demonstrated the relationship between BPH and lower urinary tract symptoms (LUTS) [2-4], and the incidence of both urological disorders increases with age [2,5]. The LUTS secondary to BPH, is caused by the urinary obstruction, leading to various storage symptoms and voiding symptoms. Two medical treatment agents (the 5alpha-reductase inhibitors (5αRIs), dutasteride and finasteride and four alpha 1-adrenergic receptor blockers, or α1-blockers, tamsulosin, alfuzosin, doxazosin and terazosin) are currently used to treat BPH and LUTS [6-7]. 5αRIs inhibit the conversion of testosterone into DHT, thereby lowering the DHT concentration and the prostatic volume. α1-blockers attenuate the urinary tract problems by relieving the contractions of the urethra, the urinary bladder neck and the prostatic smooth muscle, thereby ameliorating the urine outflow rate. Treatment of BPH with 5αRIs either alone or in combination with α1-blockers is effective, but these agents are limited because of their undesired harmful effects on the reproductive system [8-9]. Therefore, phytotherapeutic agents are now a popular alternative for treatment of BPH.

Caesalpinia sappan L. and *Uvaria rufa* Blume are plants belonging to the Leguminosae and Annonaceae families. Both of them are distributed in tropical areas, especially Southeast Asia [10-11]. *C. sappan* is commonly understood by Thai people as *phang* while *U. rufa* is known as *pee paun noi*. Various plants in the Leguminosae and Annonaceae families have been used by local Thai people as folk medicines for the treatment of urological disorders and prostatic diseases [12-16]. In addition, the heartwood of *C. sappan* is used to make phytotherapeutic agents to treat skin infections, inflammation, analgesic diarrhea, hypoglycemia, anemia and tuberculosis [17-20]. The relaxant effect on rats' aortic rings of a methanolic extract from *C. sappan* heartwood was previously reported [21]. Moreover, brazilin and hematoxylin isolated from the heartwood of *C. sappan* also exhibited relaxant effects on isolated rats' aorta [22]. The aqueous decoction from the roots and the heartwood of *U. rufa* are used to remedy fever [23]. The fruits of *U. rufa* are used against skin allergies and gastrointestinal abscesses [24]. Oh et al. (1998) revealed that the heartwood of *C. sappan* possessed high amounts of three phytosterols, campesterol, stigmasterol and beta-sitosterol [25]. A variety of phenolic compounds, including xanthone, coumarin, chalcones, flavones, isoflavonoids and brazilin, were found in the wood of *C. sappan* [15]. Various parts of *U. rufa* also contained flavonoids, flavonols, alkaloids, and flavonolrutin, isoquercitrin, kaempferol, quercitrin and lignan glycoside [10-11,26-27]. β-sitosterol has been detected in ethyl acetate extracts from *U. rufa* stems [28]. Different types of flavonoids and sterols derived from various plant materials possessed α1-adrenergic receptor antagonists and exhibited relaxation effects on the dynamic component in the prostate gland of experimental animals [29-32]. Although there is a lot of research being done on the phytochemical composition of *C. sappan* and *U. rufa*, there is no detailed information about their relaxant properties on the prostate smooth muscles. We therefore investigated the relaxant efficacy of the extracts from the stems of *C. sappan* and *U. rufa* on rats' prostatic tissues.

2. MATERIAL AND METHODS

2.1 Chemicals

Gallic acid, quercetin and tamsulosin hydrochloride, were bought from Sigma-Aldrich (St. Louis, USA). β-sitosterol (HPLC grade) was purchased from United States Biological (MA, USA). Folin & Ciocalteu's Solution was obtained from Loba Chemie, Pvt, Ltd. (Mumbai, India). Analytical grade of reagents and chemicals was used.

2.2 Plant Collection and Extraction

Caesiapinia sappan L. was acquired from Chiang Mai Province while *Uvaria rufa* Blume was acquired from Buriram Province, Thailand, in March 2014. They were identified by the botanist at the herbarium of the Queen Sirikit Botanical Garden, Thailand, where the voucher specimens under the reference numbers QSBG No. 87144 (*C. sappan*) and QSBG No. 78882 (*U. rufa*) were deposited. The stems of the plants were washed, chopped and air dried. The dried stems were pulverized. The powdered materials (100 g) were refluxed with 1,000 ml of petroleum ether in a Soxhlet apparatus followed by refluxing with ethyl acetate and 95 % ethanol respectively. Each obtained extracts were filtered. The solvents were eliminated from the filtrates under reduced pressure using a rotary evaporator (IKA® RV, China). All of the extracts were dried using a hot-air oven (Daihan Labtech, India), and they were stored at 4 ℃. The percentage of extraction yields of the ethyl acetate and ethanolic extracts from *U. rufa* and *C. sappan* were 1.42 (UEA) and 7.06 (UEOH), 1.84 (CEA) and 5.75 (CEOH), respectively.

Comment [U2]: *Caesalpinia*

Comment [U3]: what size is the mesh

2.3 Experimental Animals

Experiments were conducted using 12-week-old male albino rats (250-300 g) and obtained from the National Laboratory Animal Center, Nakorn Pathom Province, Thailand. Animals were housed and acclimatized in a standard environmentally-controlled laboratory for at least one week prior to the experiments. The room temperature was controlled at 25 ± 1 ℃ under a 12 hrs light/12 hrs dark cycle with access to a standard diet and water *ad libitum*. All of the animal procedure used in the present study were carried out in accordance with the reviewed and approved by the Institutional Animal Care and Use Committee in the Department of Biology, Faculty of Science, Chiang Mai University (ID: Re. 004/13).

2.4 Determination of α1-Adrenergic Antagonist Activity

2.4.1 Preparation of prostate tissue strip

Male Wistar rats were sacrificed and their ventral prostate lobes were surgically excised as previously described [33]. The prostatic tissues were placed in solution of Krebs-Henseleit, pH 7.4. The excessive fat and connective tissue were carefully removed. The prostate strip of approximately 10 x 5 mm was mounted in an organ bath chamber filling Krebs-Henseleit solution pre-warmed to 37 ^{o} C and continuously aerated with 5 % CO_2 in O_2. One end of a tissue strip was attached with a tissue holder while the other end was connected to a transducer. The contraction of prostatic tissue strip was measured with a SS12LA variable range force transducer connected to the Biopac Student Lab PRO® 3.7 Software (Harikul Science Co., Ltd., Thailand). The prostate strip was equilibrated for 1 hr under 1.0 g of resting tension until a baseline was attained. Fresh bath medium was replaced every 30 min. To determine the viability of the prostate tissue strip, contractions were produced by electrical field stimulation (0.5 ms, 60 V, 0.01 Hz).

2.4.2 Exogenously Administered Agonist

To measure the postjunctional effects of the extracts, adrenaline (α1-adrenoceptor agonist) was used to induce smooth muscle contractions. The concentration-response curves to adrenaline (0.001-100 µM) were constructed on each prostate strip after 60 min of stabilization. When the maximal contractile response for each concentration of adrenaline was reached, prostatic tissue was then exposed to an extract at a concentration of 250 µg/ml for 30 min. After the concentration response curve was completed once, the tissue was washed with a fresh bath medium and allowed to rest for 30 min prior to a second concentration response curve was plotted [31,33-34]. The concentration response curves produced by the extracts or control were plotted in parallel on a pair of the prostatic lobes from the same animal. A positive curve from an alpha 1-blocker (tamsulosin 50 µg/ml) was plotted at the same procedure.

2.4.3 Measurement of Prostatic Relaxation Caused by Various Extracts

To evaluate the efficacy of various extracts from *C. sappan* and *U. rufa*, which produce prostatic relaxation, a prostate strip was induced to contract by adrenaline at a concentration of 10 µM. After the maximal contraction was achieved, each extract was added in increasing concentrations ranging from 50-250 µg/ml for 10 min each. The same procedure was carried out for tamsulosin at concentrations ranging from 50-250 µg/ml [31,33-34]. The extract concentrations of *C. sappan* and *U. rufa* used in the present study were based on our previous investigation. The prostatic relaxation was expressed as percentage inhibition from the maximal contraction. Percent relaxation, percent maximal effect (E_{max}), and effective concentration of compound to produce 50 % of relaxation (EC_{50}), were determined.

2.5 Phytochemical Studies

2.5.1 Preliminary Phytochemicals

Preliminary phytochemical investigation was done by detecting the occurrence of the eleven active compounds in the various extracts following the standard methods previously described [35-36].

2.5.2 Total Phenolics

The contents of total phenolics presented in the extracts of *C. sappan* and *U. rufa* was determined using a Folin-Ciocalteu reagent. Briefly, 0.1 ml of each extract was added to 2.0 ml of Folin-Ciocalteu reagent and then sodium carbonate (7.5 % w/v) reagent. The absorbance was measured at 760 nm by a spectrophotometer after 20 min of incubation at 25 ºC [37]. The analysis was carried out in triplicate. The standard calibration curve was made from gallic acid.

2.5.3 Total Flavonoids

The content of flavonoids in the extracts of *C. sappan* and *U. rufa* was determined using the protocol of [38]. Briefly, 0.1 ml of each extract was added to aluminium chloride solution (0.5 ml) and incubated at room temperature for 60 min. The intensity of yellow color was measured at 420 nm. The analysis was done in triplicate. The standard calibration curve was made from quercetin.

2.6 Data Analysis

All data was represented as mean ± standard error of mean (S.E.M). One-way ANOVA, followed by Duncan's post hoc test for multiple comparisons was used to analyze a significant difference between control and treated groups. All graphs and data were analyzed using GraphPad Prism, Version 7.0 for Windows. The EC_{50} values were analyzed using linear regression. A student's t-test was used to measure a significant difference between agonist and antagonist. The values of $P < 0.05$ or $P < 0.01$ or $P < 0.001$ are considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1 Effects of Various Extracts on Contractile Responses to Adrenaline

From the organ bath studies, we knew that administration of adrenaline in concentrations ranging from -9 to -4 M (0.001-100 µM) induced prostate contractions in a concentration-dependent manner (Fig.1). The forces of prostate contraction were reduced following incubation of CEA, CEOH, UEA and UEOH extracts at 250 µg/ml, for 30 min (Fig.1). Both UEA and UEOH extracts strongly relaxed the prostate smooth muscle contraction induced by adrenaline at -7 to -4 M. The CEA and CEOH extracts exhibited relaxant efficacy less than the UEA and UEOH extracts. The contractile responses to adrenaline at -8 to -4 M were significantly reduced ($P < 0.001$) following the incubation of tamsulosin (50 µg/ml). At a concentration of -4 M of adrenaline, the UEOH extract had the highest ability to reduce the force of contraction. The order was as follows: UEOH (0.34 ± 0.09 g), UEA (0.33 ± 0.03 g), CEA (0.25 ± 0.09 g) and CEOH (0.13 ± 0.07 g). At the same concentration of adrenaline, the reduction produced by tamsulosin was 0.61 ± 0.03 g (Fig.1).

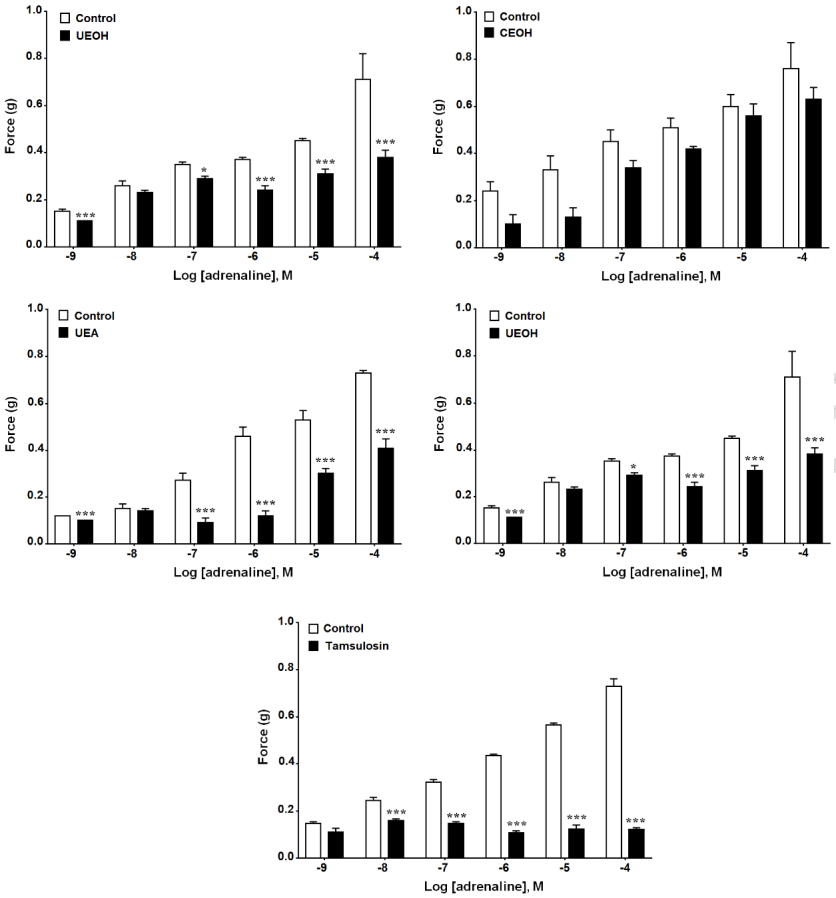

Fig. 1. Effects of CEA, CEOH, UEA and UEOH extracts at 250 µg/ml, and tamsulosin at 50 µg/ml, on adrenaline-induced contractions of isolated rats' prostate strips

*Force is expressed as mean ± standard error of means. * P < 0.05, *** P < 0.001 as compared with control (Student's t-test)*

3.2 Relaxant Efficacy of Various Extracts on Isolated Rats' Prostate Strips

The contractile responses to various extracts were investigated in the rats' prostate smooth muscles pre-contracted by adrenaline (10 µM). As shown in Fig.2, all of the extracts, as well as tamsulosin (50-250 µg/ml), significantly reduced ($P < 0.001$) prostate contraction induced by adrenaline. At every concentration, the UEA extract exhibited the most potency in relaxing the prostatic smooth muscle.

All of the extracts as well as tamsulosin exhibited relaxant effects on isolated prostate smooth muscles in a concentration-dependent manner (Fig.3). The UEA extract at concentrations ranging from 50-250 µg/ml were more potent in relaxing the prostate smooth muscle than other extracts. At 250 µg/ml, the order of maximal effects of various extracts is thus UEA (72.09 ± 2.15 %) > CEA (55.59 ± 2.90 %) > CEOH (54.50 ± 1.18 %) > UEOH (51.35 ± 4.42 %). However, all extracts had a lower relaxant efficacy than tamsulosin. The EC_{50} value of the UEA extract was 140.23 ± 9.74 µg/ml while that of tamsulosin was 86.83 ± 8.96 µg/ml (Table 1).

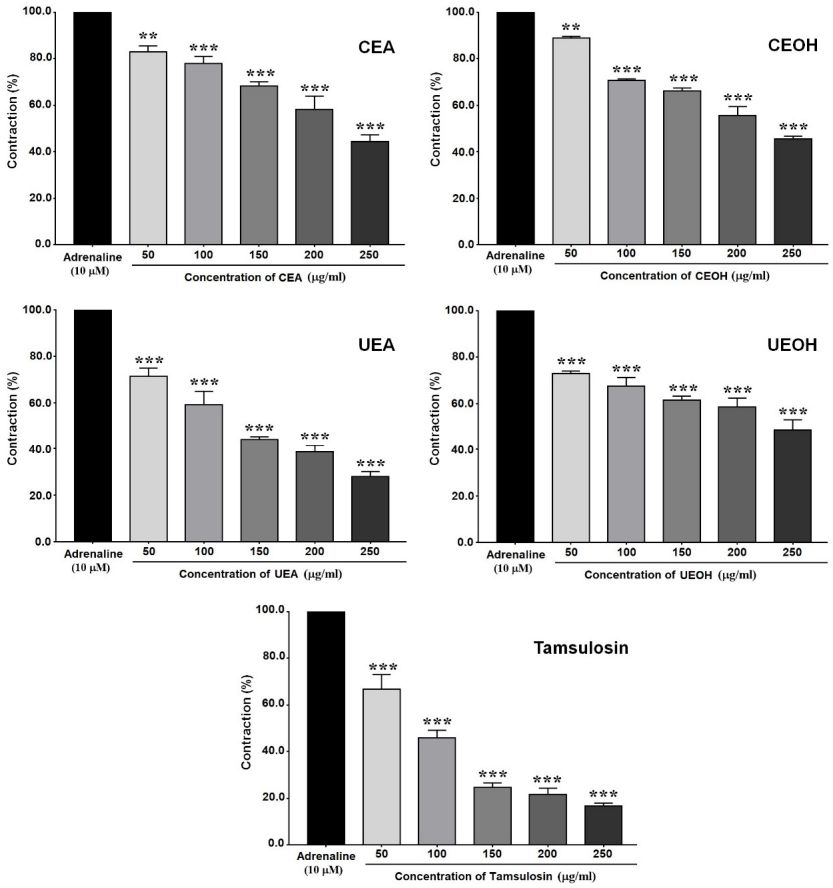

Fig. 2. Effect of CEA, CEOH, UEA, UEOH and tamsulosin at 50-250 µg/ml on adrenaline-induced contraction of isolated rats' prostate strips

*Data are expressed as mean ± standard error of means. ** P < 0.01, *** P < 0.001 as compared with control (Student's t-test)*

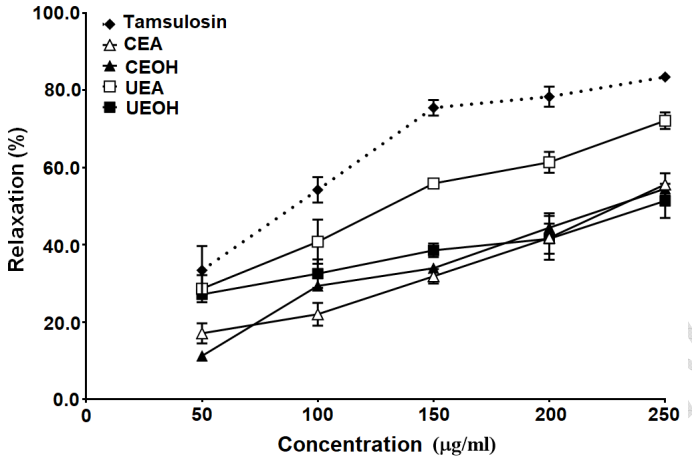

Fig. 3. Relaxant effects of various extracts and tamsulosin on isolated prostate strips
Data are expressed as mean ± standard error of means.

Table 1. Maximal effect (E_{max}) and EC_{50} values of various extracts in relaxing adrenaline-induced prostate contraction

Sample	E_{max} (%)	EC_{50} (µg/ml)
CEA	55.59 ± 2.90	236.24 ± 5.05
CEOH	54.50 ± 1.18	226.35 ± 7.16
UEA	72.09 ± 2.15	140.23 ± 9.74
UEOH	51.35 ± 4.42	235.35 ± 24.96
Tamsulosin	83.42 ± 1.32	86.83 ± 8.96

Data are represented as mean ± standard error of means.

3.3 Phytochemical Studies

From preliminary phytochemical analysis, various phytochemical compounds, including flavonoids, phenolics, sterols, tannins, phlobatannins, terpenoids, cardiac glycosides, alkaloids, saponins, anthraquinones and reducing sugars were found in the UEOH extract. The CEA, CEOH and UEA extracts possessed the phytochemical compounds we tested for, except saponins in the CEA extract and except anthraquinones in the CEOH and UEA extracts (Table 2).

Table 2. Phytochemical constituents of various extracts from *C. sappan* and *U. rufa*

Compounds	Phytochemical results			
	CEA	CEOH	UEA	UEOH
Phenolics	+	+	+	+
Flavonoids	+	+	+	+
Tannins	+	+	+	+
Phlobatannins	+	+	+	+
Terpenoids	+	+	+	+
Alkaloids	+	+	+	+

Sterols	+	+	+	+
Saponins	-	+	+	+
Anthraquinones	+	-	-	+
Cardiac glycosides	+	+	+	+
Reducing sugars	+	+	+	+

Note: + present, - absent

The highest quantity of total phenolics and total flavonoids were found in the CEOH and CEA extracts respectively. The order of phenolics was as follows: CEOH > CEA > UEA > UEOH with the values 22.68 ± 1.53, 21.39 ± 0.34, 13.97 ± 0.43 and 6.77 ± 0.11 mgGAE/g extract respectively (Fig.4). The order of flavonoids is thus CEA > CEOH > UEA and UEOH with the values of 12.89 ± 0.15, 11.33 ± 0.20, 3.85 ± 0.25 and 1.51 ± 0.05 mgQE/g extract respectively (Fig.5).

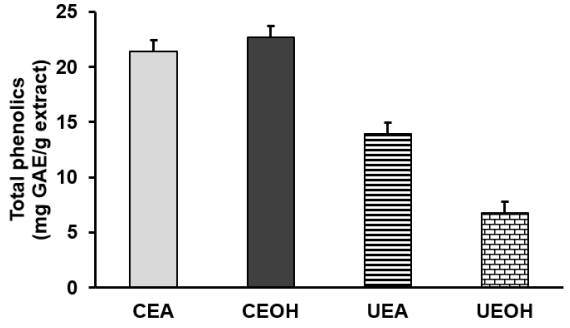

Fig. 4. Amounts of total phenolics in various extracts
Data are represented as mean ± standard error of means.

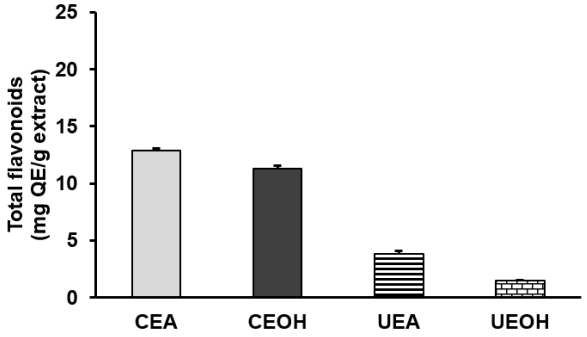

Fig. 5. Amounts of total flavonoids in various extracts
Data are represented as mean ± standard error of means.

4. DISCUSSION

Since alpha-1 adrenoceptors play an essential role in controlling the function of smooth muscles in the prostate and lower urinary tract, one of the most effective agents used to treat BPH and urological symptoms is the α_1-adrenoceptor antagonist (alpha-1 blocker). Previous investigation showed that alpha-1 blockers were able to relax isolated prostate contraction in mice [39-40] and rats [41]. Therefore, agents which are able to relax the tone of rodent prostate smooth muscles via the same mechanisms as found in the human prostate, may relieve BPH symptoms. Although alpha-1 blockers effectively treat voiding symptoms secondary to BPH, they produce adverse side effects on the reproductive system. Various compounds derived from plant materials are now popular alternatives as anti-BPH agents. Therefore, this study investigated whether *C. sappan* and *U. rufa* were able to inhibit rats' prostatic smooth muscle contractions.

Ventral prostate lobe of human and rodents was widely used as a model in laboratory for investigating relaxant efficacy of various agents [31,33-34]. In this study, we used rats' prostatic smooth muscle for testing relaxant efficacy of extracts from the stems of *C. sappan* and *U. rufa*. Our results showed that the UR-EtOAc, UR-EtOH, CS-EtOAc, and CS-EtOH extracts effectively reduced the contraction of prostatic smooth muscles induced by adrenaline at concentrations of -9 to -4 M. All of the extracts at concentrations ranging from 50-250 µg/ml caused a concentration-dependent relaxation in isolated rats' prostates pre-contracted with adrenaline. The UR-EtOAc was more potent as a relaxant agent against prostate contraction than other extracts. The relaxant properties of these extracts were consistent with the therapeutically beneficial action of tamsulosin. However, all extracts had a lower relaxant ability than tamsolusin. The therapeutic action of the extracts against the smooth muscle contraction produced by adrenaline may come from various mechanisms. Tamsulosin antagonizes the impact of adrenergic neurotransmitters at the α_1-adrenoceptor, and reduces the tones of smooth muscle cells in the prostatic stroma, prostatic urethra and the bladder neck, leading to improved voiding symptoms [42-43]. Thus, the extracts may act at postjunctional sites of adrenergic neurons and disrupt the binding of smooth muscle receptors, especially α_1-adrenoceptors, with their signaling system [34]. The relaxant effects of the extracts from *C. sappan* and *U. rufa* detected from this study may be due to the occurrence of two bioactive compounds, phenolics and flavonoids, in these plant extracts. Isoflavones, a flavonoid from red clover (*Trifolium pratense*), exhibited a relaxant effect on the smooth muscles of isolated guinea-pigs' ilea [44], rats' uteri [45], guinea-pigs' gall bladders [46] and rats' prostate glands [31]. Furthermore, a variety of flavonoids derived from various plant materials possessed α1-adrenergic receptor antagonists and reduced the contraction of the prostate smooth muscles of experimental animals [29-32].

In this study, besides the efficacy of flavonoids presented in *C. sappan* and *U. rufa*, the phytosterols in the extracts from these plants may have acted as alpha-1 blockers and alleviated the prostate contractions produced by adrenaline. Phytochemical investigation found the presence of sterols in all four extracts. An important phytosterol, β-sitosterol, has previously been detected in ethyl acetate extracts from *U. rufa* stems [28]. The heartwood of *C. sappan* possessed high amounts of three phytosterols, campesterol, stigmasterol and beta-sitosterol [25]. An earlier study confirmed the potential role of β-sitosterol to manage BPH in human and animal models. Wilt et al. (1999) reported that β-sitosterol at a dose of 10 mg/kg could improve urological symptoms and urine flow rate in BHP men [47]. The relaxant effect of phytosterols from flaxseed, fruits of saw palmetto, and red clover on isolated prostate strips have been demonstrated [30-32]. The methanolic extract from the heartwood of *C. sappan* was able to produce relaxant effects on rats' aortic rings [21]. Moreover, two purified active compounds, brazilin and hematoxylin, from *C. sappan* heartwood also exhibited relaxant effects on isolated rats' aorta [21-22]. The various efficacies of different extracts from *C. sappan* and *U. rufa* in relaxing smooth muscles found in this study may depend on the amounts of phytochemical compounds, especially flavonoids and sterols, in each extract. Since the tone of prostate smooth muscles is regulated by various factors such as adrenergic neurons, cholinergic neurons, and nonadrenergic noncholinergic neurotransmitters, and endogenous factors from vascular endothelial sources [48], the relaxant effect produced by plant extracts on smooth muscle contraction may be caused by other mechanisms. The relaxant effects of saw palmetto, which altered the release of neurotransmitters from the nerves which supplied the rat prostate were reported [49]. In addition, activation of BK_{Ca} (Large-conductance Ca^{2+}-activated K^+ channels) also caused the relaxation of smooth muscles via hyperpolarization of the membrane potential [50]. The vasorelaxant activity of brazilin isolated from *C. sappan* on isolated rat aorta and umbilical vein endothelial cells has been proven [22]. This compound acted by increasing concentration of intracellular calcium ions in the squamous cells of vascular vessels, consequently stimulating calcium ions/calmodulin-dependent nitric oxide production. When the nitric oxide is released and delivered into the smooth muscle cells, it results in vasorelaxation. So control of prostatic contractions may be linked to these mechanisms. Therefore, further study of the specific mechanisms of the extracts from the stems of *U. rufa* in relaxing the prostate smooth muscle is needed.

5. CONCLUSION

We concluded that the ethyl acetate and ethanolic extracts from the stems of *C. sappan* and *U. rufa* exhibited relaxant effects against adrenaline-induced rats' prostate strip contractions. The ethyl acetate from the stems of *U. rufa* was the most effective relaxant agent. Therefore, this extract may be useful to relieve the urological symptoms caused by BPH.

COMPETING INTERESTS

Authors have declared that no competing interests exists.

CONSENT

It is not applicable.

Comment [U4]: should be consistent with the above CEA, CEOH, UEA, UEOH

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the ethics committee in the Department of Biology, Faculty of Science, Chiang Mai University. The approval number is Re. 004/13.

REFERENCES

Comment [U5]: there are 30 references (60%) that are not up to date (more than 10 years)

1. Roehrborn CG. Lower urinary tract symptoms, benign prostatic hyperplasia, erectile dysfunction, and phosphodiesterase-5 inhibitors. Rev Urol. 2004; 6(3):121-127.
2. Roehrborn CG. Pathology of benign prostatic hyperplasia. Int J Impot Res. 2008;20(3):S11-S18.
3. Oelke M, Giuliano F, Mirone V, Xu L, Cox D, Viktrup L. Monotherapy with tadalafil or tamsulosin similarly improved lower urinary tract symptoms suggestive of benign prostatic hyperplasia in an international, ramdomised, parallel, placebo-controlled clinical trial. Eur Urol. 2012;61(5):917-25.
4. Gacci M, Andersson KE, Chapple C, Maggi M, Mirone V, Oelke M, et al. Latest evidence on the use of phosphodiesterase type 5 inhibitors for the treatment of lower urinary tract symptoms secondary to benign prostatic hyperplasia. Eur Urol. 2016;70(1):124-33.
5. Rosen R, Altwein J, Boyle P, Kirby RS, Lukacs B, Meuleman E, et al. Lower urinary tract symptoms and male sexual dysfunction: the Multinational Survey of the Aging Male (MSAM-7). Eur Urol. 2003;44(6):637-49.
6. Montorsi F, Moncada I. Safety and tolerability of treatment for BPH. Eur Urol Suppl. 2006;5(20):1004-1012.
7. Aggarwal S, Thareja S, Verma A, Bhardwaj TR, Kumar M. An overview on 5α-reductase inhibitors. Steroids. 2010;75(2):109-53.
8. MacDonald R, Wilt TJ, Howe RW. Doxazosin for treating lower urinary tract symptoms compatible with benign prostatic obstruction: a systematic review of efficacy and adverse effects. BJU Int. 2004;94(9):1263-70.
9. Traish AM, Hassani J, Guay AT, Zitzmann M, Hansen ML. Adverse side effects of 5α-reductase inhibitors therapy: persistent diminished libido and erectile dysfunction and depression in a subset of patients. J Sex Med. 2011;8(3):872-84.
10. Deepralard K, Kawanishi K, Moriyasu M, Pengsuparp T, Suttisri R. Flavonoid glycosides from the leaves of *Uvaria rufa* with advanced glycation end-products inhibitory activity. Thai J Pharm Sci. 2009;33(2/3):84-90.
11. Nguyen TH, Ho VD, Do TT, Bui HT, Phan VK, Sak K. et al. A new lignan glycoside from the aerial parts and cytotoxic investigation of *Uvaria rufa*. Nat Prod Res. 2015;29(3):247-52.
12. Rojas R, Bustamante B, Bauer J. Antimicrobial activity of selected Peruvian medicinal plants. J Ethnopharmacol. 2003;88(2-3):199-204.
13. Ogueke CC, Ogbulie JN, Anyanwu BN. The effects of ethanolic and boiling water extracts of root barks and leaves of *Uvaria chamae* on some hospital isolates. J Am Sci. 2007;3(3):68-73.
14. Agbodjogbe WKDD, Aïkpe JFA, Ayedoun MA, Assogba FM, Dansou PH, Gbenou JD. Diuretic and natriuretic activities from ten medicinal plants used in south Benin. J Chem Pharm Res. 2015;7(12):1145-1152.
15. Nirmal NP, Rajput MS, Prasa RGSV, Ahmad M. Brazilin from *Caesalpinia sappan* heartwood and its pharmacological activities: a review. Asian Pac J Trop Med. 2015;8(6):421-30.
16. Yovo M, Alitonou GA, Yedomonhan H, Tchobo F, Dedome O, Sessou P, et al. First report on chemical composition and antimicrobial activity of *Artabotrys velutinus* Scott-Elliot extracts against some clinical strains in Benin. Am J Appl Chem. 2016;4(3):71-76.
17. Xu HX, Lee SF. The antibacterial principle of *Caesalpinia sappan*. Phytother Res. 2004;18(8):647-51.
18. You EJ, Khil LY, Kwak WJ, Won HS, Chae SH, Lee BH, et al. Effects of brazilin on the production of fructose-2,6-bisphosphate in rat hepatocytes. J Ethnopharmacol. 2005;102(1):53-7.
19. Washiyama M, Sasaki Y, Hosokawa T, Nagumo S. Anti-inflammatory constituents of Sappan lignan. Biol Pharma Bull. 2009;32(5):941-4.
20. Sireeratawong S, Piyabhan P, Singhalak T, Wongkrajang Y, Temsiririrkkul R, Punsrirat J, et al. Toxicity evaluation of sappan wood extract in rats. J Med Assoc Thai. 2010;93(suppl7): S50-7.
21. Xie YW, Ming DS, Xu HX, Dong H, But PPH. Vasorelaxing effects of *Caesalpinia sappan*: involvement of endogenous nitric oxide. Life Sci. 2000;67(15):1913-8.
22. Hu CM, Kang JJ, Lee CC, Li CH, Liao JW, Cheng YW. Induction of vasorelazation through activation of nitric oxide synthase in endothelial cells by brazilin. Eur J Pharmacol. 2003;468(1): 37-45.
23. Nanakorn W. Queen Sirikit Botanic Garden. 5th ed. Bangkok: Printing House; 1998.
24. Rosandy AR, Din LB, Yaacob WA, Yusoff NI, Sahidin I, Latip J, et al. Isolation and characterization of compounds from the stem bark of *Uvaria rufa* (Annonaceae). Malaysian J Anal Sci. 2013;17(1):50-58.

25. Oh RS, Kim DS, Lee IS, Jung KY, Lee JJ, Lee HK. Anticomplementary activity of constituents from the heartwood of *Caesalpinia sappan*. Planta Med. 1998;64(5):456-8.
26. Tip-pyang S, Payakarintarungkul K, Sichaem J, Phuwapraisirisan P. Chemical constituents from the roots of *Uvaria rufa*. Chem Nat Compd. 2011;47(3):475-476.
27. Paragas EM, Gehle D, Krohn K, Franzblau SG, Macabeo APG. Anti-tubercular flavonol derivatives from *Uvaria rufa*. Res J Pharm Biol Chem Sci. 2014;5(6):856-859.
28. Buncharoen W, Saenphet K, Saenphet S, Thitaram C. *Uvaria rufa* Blume attenuates benign prostatic hyperplasia via inhibiting 5α-reductase and enhancing antioxidant status. J Ethnopharmacol. 2016;194(2016):483-4.
29. Ajay M, Gilani AH, Mustafa MR. Effects of flavonoids on vascular smooth muscle of the isolated rat thoracic aorta. Life Sci. 2003;74(5):603-12.
30. Zhang HL, Tang ZY, Yang JX, Zhang Y, Li Y, Lin Y. Bi-directional regulation of emodin and quercetin on smooth muscle myosin of gizzard. FEBS Lett. 2006;580(2):469-73.
31. Brandli A, Simpson JS, Ventura S. Isoflavones isolated from red clover (*Trifolium pretense*) inhibit smooth muscle contraction of the isolated rat prostate gland. Phytomedicine. 2010;17(11):895-901.
32. Chua T, Eise NT, Simpson JS, Ventura S. Pharmacological characterization and chemical fractionation of a liposterolic extract of saw palmetto (*Serenoa repens*): Effects on rat prostate contractility. J Ethnopharmacol. 2014;152(2):283-91.
33. Choo SH, Sung HH, Chae MR, Kang SJ, Han DH, Park JK, et al. Effects of *Schisandra chinensis* extract on the relaxation of isolated human prostate tissue and smooth muscle cell. J Ethnopharmacol. 2014;156(2014):271-6.
34. Xu Y, Ventura S. Extracts of bark from the traditional Chinese herb *Phellodendron amurense* inhibit contractility of the isolated rat prostate gland. J Ethnopharmacol. 2010;127(1):196-9.
35. Edeoga HO, Okwu DE, Mbaebie BO. Phytochemical constituents of some Nigerian medicinal plants. Afr J Biotechnol. 2005;4(7):685-688.
36. Ayoola GA, Coker HAB, Adesegun SA, Adepoju-Bellol AA, Obaweyal K, Ezennial EC, et al. Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in Southwestern Nigeria. Trop J Pharm Res. 2008;7(3):1019-1024.
37. Wolfe K, Wu X, Liu RH. Antioxidant activity of apple peels. J Agric Food Chem. 2003;51(3):609-14.
38. Ordonez AAL, Gomez JD, Vattuone MA, Isla MI. Antioxidant activities of *Sechium edule* (Jacq.) Swart extracts. Food Chem. 2006;97(3):452-458.
39. Gray K, Short J, Ventura S. The alpha1A-adrenoceptor gene is required for the alpha1L-adrenoceptor-mediated response in isolated preparations of the mouse prostate. Br J Pharmacol. 2008;155(1):103-9.
40. Gray KT, Ventura S. Alpha1L-adrenoceptors mediate contractions of the isolated mouse prostate. Eur J Pharmacol. 2006;540(1-3):155-61.
41. Ventura S, Dewalagama RK, Lau LC. Adenosine 5'-triphosphate (ATP) is an excitatory cotransmitter with noradrenaline to the smooth muscle of the rat prostate gland. Br J Pharmacol. 2003;138(7):1277-84.
42. Caine M, Pfau A, Perlberg S. The use of alpha-adrenergic blockers in benign prostatic obstruction. Br J Urol. 1976;48(4):255-63.
43. Monda JM, Oesterng JE. Medical treatment of benign prostatic hyperplasia: 5 alpha-reductase inhibitors and alpha-adrenergic antagonists. Mayo Clin Proc. 1993;68(7):670-9.
44. Herrera MD, Marhuenda E, Gibson A. Effects of Genistein, an isoflavone isolated from *Genista tridentate*, on isolated guinea-pig ileum and guinea-pig ileal myenteric plexus. Planta Med. 1992;58(4):314-6.
45. Revuelta MP, Cantabrana B, Hidalgo A. Depolarization-dependent effect of flavonoids in rat uterine smooth muscle contraction elicited by $CaCl_2$. Gen Pharmacol Vasc S. 1997;29(5):847-57.
46. Wang LD, Qiu XQ, Tian ZF, Zhang YF, Li HF. Inhibitory effects of genistein and resveratrol on guinea pig gallbladder contractility *in vitro*. World J Gastroenterol. 2008;14(31):4955-4960.
47. Wilt TJ, MacDonald R, Ishani A. Beta-sitosterol for the treatment of benign prostatic hyperplasia: a systematic review. Br J Urol Int. 1999;83(9):976-83.
48. Giuliano F, Ückert S, Maggi M, Birder L, Kissel J, Viktrup L. The mechanism of action of phophodiesterase type 5 inhibitors in the treatment of lower urinary tract symptoms related to benign prostatic hyperplasia. Eur Urol. 2013;63(3):506-16.
49. Cao N, Haynes JM, Ventura S. Saw palmetto is an indirectly acting sympathomimetic in the rat-isolated prostate gland. Prostate. 2006;66(2):115-23.
50. Lang RJ, Mulholland E, Exintaris B. Characterization of the ion channel currents in single myocytes of the guinea pig prostate. J Urol. 2004;172(3):1179-87.