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Asian Medical Journal and Alternative Medicine (AMJAM) aimed to publish high quality, peer-review articles. This journal publishes Original article, Review article, Special article, Brief research, Case report, and Letter to the editor in all health science medicine specialties and alternative medicine. Manuscripts submitted to AMJAM should consist of new material that focuses on broad topics in health science published in English language. AMJAM published 4 issues annually (January-April, May-August, September-December, and 1 supplemental issues per year). All submitted article will be evaluated by double-blinded review process by 2-4 reviewers.

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Office Address Research Administration Office, Faculty of Medicine, Thammasat University (Rangsit Campus)
Klongluang, Pathum Thani 12120, Thailand
Tel. +66 2564 4444 ext.7530-39
Email: amjam.journal@yahoo.com
Website http://www.asianmedjam.com

Office of Publication Thammasat Printing House 2022, Klongluang, Pathum Thani 12120, Thailand
Tel. +66 2564 3104-6 Fax. +66 2564 3119
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Editor's Note

As always, AMJAM offers interesting articles related to the field of medicine and alternative medicine. We offer a mixed of medical and alternative sciences in this issue. With the increasing numbers of submissions, we believe that the quality of AMJAM is now improved and several articles were submitted from another countries. Together, these changes reflect the right path of the journal going into the Scopus Index. AMJAM quality rely on a high quality of submissions from readers and academician and we really hopefully that the changes in the year to come including partnership with Elsevier Publisher will enhance submission, citation and visibility of our works in scientific community. Editorial team would like to express our sincere thank you to all reviewers and authors that significantly improved the quality of our journal.

Anucha Apisarnthanarak, M.D.
Professor in Infectious Diseases
Editor-in-Chief
Asian Medical Journal and Alternative Medicine (AMJAM)

Editorial

Recipe 2, A Thai Herbal Remedy in the Worayokasarn Scripture, for Reducing Obesity

Pannawat Chaiyawatthananthn*

Recipe 2 is a Thai herbal medical remedy for treating obesity in the Worayokasarn scripture. The recipe consists of three plants: *Terminalia chebula* Retz. (Sa-Mor-Thai) fruits, *Cyperus rotundus* L. (Hua-Haw-Mhoo) rhizomes, and *Tinospora crispa* (L.) Miers ex Hook. f. & Thomson or *Tinospora cordifolia* (Thunb.) Miers (Bo-Ra-Pet) vines.[1] The three herbal ingredients of the remedy showed anti-obese effects by reducing lipid accumulation and lipogenesis. The ethanolic extract of *T. chebula* fruit decreased lipogenesis in obese mice by reducing fatty acid synthase, and increasing fatty acid oxidation through peroxisome proliferator-activated receptors, α (PPARα) and carnitine palmitoyltransferase-1 (CPT-1).[2] After 14 days of treatments, Haritaki or *T. chebula* at 1.05 and 2.10 mg/kg body weight (b.w.) concentrations reduced total cholesterol (TC) and total triglycerides (TG) in hyperlipidemia-induced Wistar rats with an atherogenic diet.[3] Majeed and coworker's presented that Piceatannol, Scirpusin A and Scirpusin B in the *C. rotundus* rhizome extract reduced adipogenesis in 3T3-L1 adipocytes.[4] 450 mg/kg concentrations of aqueous *T. crispa* extract in 0.5% cholesterol chow diet-inducing New Zealand White rabbits showed an effect in reducing blood serum TC, TG and low density lipoprotein-cholesterol (LDL-C) levels, while increasing high density lipoprotein-cholesterol (HDL-C) levels.[5] After the feeding of a high fat diet in rats for 12 weeks, leading to the development of obesity, the obese rats receiving *T. cordifolia* stem powder at 1 mg/g b.w. for 12 weeks had subsided body weight, adiposity index, serum cholesterol, and serum triglyceride.[6]

These scientific studies show the anti-obese effects of the herbal remedy ingredients: reducing lipid accumulation and reducing serum lipids. This remedy may be effective to reduce obesity and improve lipid profile following traditional Thai medical theory.

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Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathum Thani 12120, Thailand
***Corresponding author:** Pannawat Chaiyawatthananthn, Ph.D., Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathum Thani 12120, Thailand, Email: pannawat@tu.ac.th

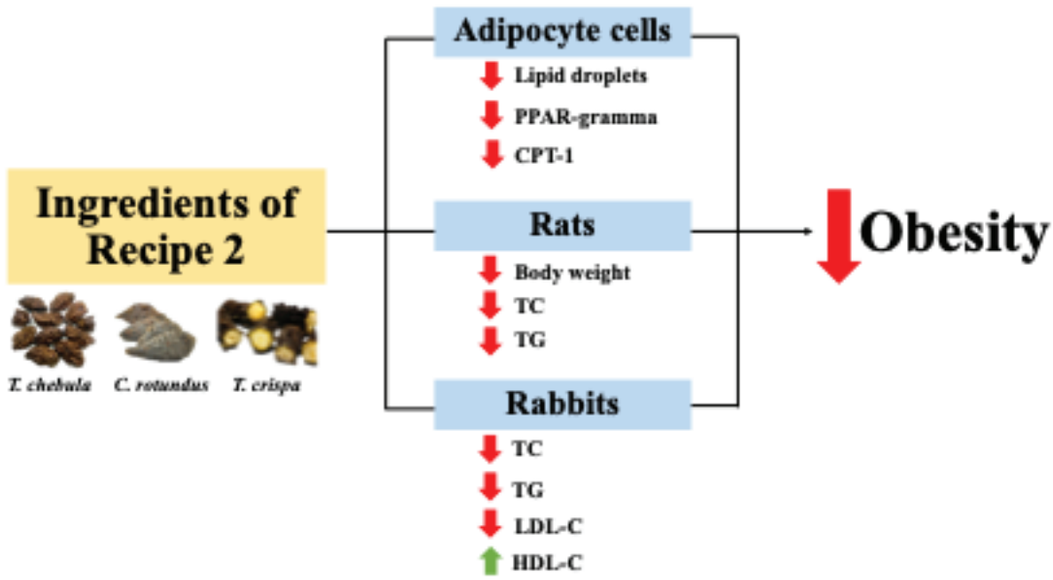

Figure 1 Lipid-lowering activities of the ingredients of Recipe 2 in Worayokasarn scripture.

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Original Article

Chest Specialists versus Non-Specialists following International COPD Recommendations in Real-World Clinical Practice

Tinn Kiatchotchai, Narongkorn Saiphoklang*

Abstract

Introduction: Real-world clinical practice for management of chronic obstructive pulmonary disease (COPD) differs from international recommendations by the Global Initiative for Chronic Obstructive Lung Disease and this affects clinical outcomes.

Objectives: To determine rates of following the recommendations and the impact on the clinical outcomes in COPD patients.

Methods: A prospective study was conducted in 2 outpatient clinics at a University Hospital in Thailand. Demographics and clinical data were collected. Chest specialist (CS) and non-CS groups clinical data was compared.

Results: One hundred forty-five patients (87.6% male) were included. Of these, 81 (55.9%) were followed up at a CS outpatient department. The overall prevalence of COPD management following recommendations was 29.0% (27.2% in the CS group and 31.3% in the non-CS group, P = 0.590). Compared to the non-CS group, the CS group had higher proportions of chronic kidney disease (21.0% vs 7.8%, P = 0.028), coronary heart disease (35.8% vs 15.6%, P = 0.007), and modified Medical Research Council scores (1.9 ± 1.1 vs 1.5 ± 1.2, P = 0.038). The CS group also had higher rates of vaccinations and pulse oximetry measurement than the non-CS group. There were no differences in pulmonary functions or exacerbation and hospitalization rates between the two groups.

Conclusions: One-third of patients were managed following the COPD recommendations in real-world practice. The rates of following the recommendations did not significantly differ between the CS and the non-CS groups. This finding may explain the similar clinical outcomes. Nevertheless, a larger prospective study is required to compare clinical outcomes between the two groups.

Keywords: Adherence, Clinical characteristics, COPD, GOLD recommendation, Outcomes

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Department of Internal Medicine, Faculty of Medicine, Thammasat University, Thailand
***Corresponding author:** Narongkorn Saiphoklang, Department of Internal Medicine, Faculty of Medicine, Thammasat University, Thailand, E-mail: m_narongkorn@hotmail.com

Introduction

Chronic obstructive pulmonary disease (COPD) is a common, preventable, and treatable disease which is characterized by persistent respiratory symptoms and airflow limitation.[1] It is a leading cause of the global mortality. In 2010, there was approximately 384 million people with COPD, with a global prevalence of 11.7%[1]; the prevalence of COPD in Thailand was 3.7 to 7.1%.[2, 3]

Because of COPD's severity, the Global Initiative for Chronic Obstructive Lung Disease (GOLD)[1] establishes international recommendations for COPD management to guide physicians. Diagnosing disease and grading severity of COPD has to be done carefully and appropriately to properly manage patients with COPD. Diagnosing COPD is supported by spirometry using post-bronchodilator forced expiratory volume in one second (FEV_1) to forced vital capacity (FVC) ratio less than 0.7. Moreover, spirometry is also used for classifying the degree of airway obstruction into GOLD grade 1 to 4.[1] COPD patients can be categorized into 4 groups (ABCD) depending on clinical symptoms and risk of exacerbation.[1] Nonetheless, in real-world clinical practice, the recommendations are not followed as strictly as they should be, leading to misclassification of the disease's severity and ineffective therapy, both pharmacological and non-pharmacological e.g. inhaled medication, vaccinations, pulmonary rehabilitation, which affects the clinical outcomes of COPD patients, including quality of life, acute exacerbation and hospitalization.[4-9]

Some COPD patients, such as those with severe disease, uncertain diagnosis or presence of several comorbidities, need to be properly managed by pulmonologists. Adherence to the guidelines for COPD management varies among different physicians and healthcare clinics, which might affect COPD clinical outcomes, in addition to disease severity and comorbidities. This study aimed to determine the relationship of following international COPD recommendations and the rate of COPD exacerbation, and to compare chest-specialist (CS) and non-chest-specialist (non-CS) departments.

Methods

Study design and participants

A prospective study was conducted at 2 medical outpatient departments, with 10 chest physicians and 120 non-chest physicians, at Thammasat University Hospital, Thailand between July 2020 and January 2022. Patients aged 40 years or older with 10-pack-year smoking history, and diagnosis of COPD confirmed by a physician were included. Exclusion criteria were inability to complete the questionnaires and asthma.

Ethics approval was obtained from the Human Research Ethics Committee of Thammasat University No.1 (Faculty of Medicine), Thailand (IRB No. MTU-EC-IM-0-128/63, COA No. 212/2020), in compliance with Declaration of Helsinki, The Belmont Report, CIOMS Guidelines and The International Practice (ICH-GCP). All methods were performed in accordance with these guidelines and regulations. All participants provided written informed consent.

Data collection

Patient data was collected from electronic medical records and clinical COPD questionnaires including modified Medical Research Council (mMRC), COPD Assessment Test (CAT), and the exacerbation history for 1 year. Patients' demographic data, clinical characteristics, pulmonary functions by spirometry with bronchodilator test, disease classification, and clinical outcomes (exacerbation and hospitalization due to COPD) were also recorded. Bronchodilator response is improvement in FEV_1 and/or FVC of ≥12% and ≥200 mL from baseline according to the American Thoracic Society and European Respiratory Society 2005 criteria[10]. An exacerbation in this study was defined as an acute worsening of respiratory symptoms that requires additional therapy; oral antibiotics and/or oral corticosteroids, or treatment in the emergency department or a hospitalization.

Definition of terms

For the purpose of this study, 'adherence to the COPD recommendations' was defined as compliance with both of the following criteria, which are recommended by GOLD 2019.[1]

1. Based on pulmonary function data, participants had respiratory symptoms and persistent airflow limitation (post-bronchodilator FEV_1/FVC ratio < 0.7) consistent with COPD.

2. COPD severity was classified (grade 1 to 4 and/or ABCD groups) and symptoms were assessed using CAT or mMRC.

Study outcomes

The primary outcome was proportions of physicians following the GOLD recommendations and relationships between following the recommendations and clinical outcomes (exacerbation and hospitalization) of COPD patients. The secondary outcome was comparisons of clinical outcomes and following the GOLD recommendations between the CS and the non-CS groups.

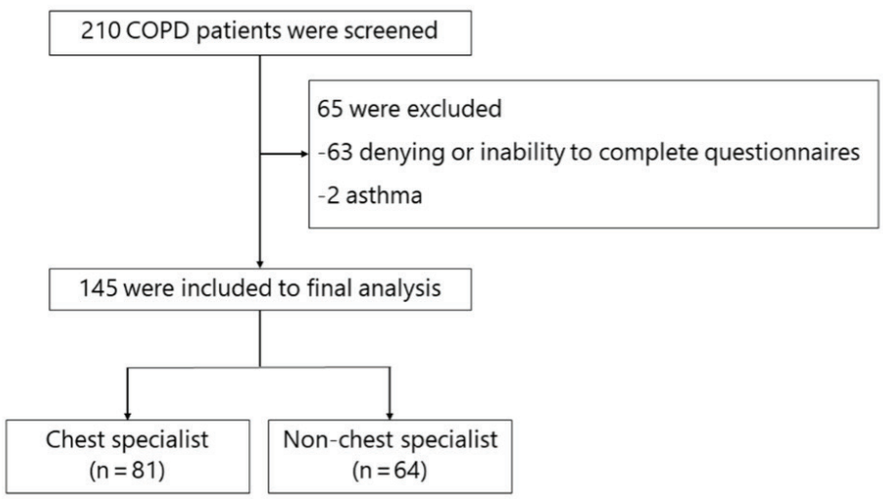
Statistical analysis

Based on a previous study[11], the prevalence of inappropriate treatment of COPD patients was 38.4%. The sample size was calculated using 80% power, 5% type I error, and 8% precision margin. Thus, the sample size would be 142.

Data is presented as number (%) and mean ± standard deviation. Chi-squared test was used to compare categorical variables between the CS and the non-CS groups. Student's t-test was used to compare continuous variables between two groups. A two-sided *p-value* < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS version 26.0 software (IBM Corp., Armonk, NY, USA).

Results

A total of 210 COPD patients were screened. Of these, 145 patients (87.6% male) were eligible for inclusion and 65 patients were excluded (Fig 1). No patient with asthma-COPD overlap was screened. Mean age was 75.03 ± 9.03 years. Post-bronchodilator FEV_1 was 66.22 ± 24.10% predicted. Most patients were in spirometry grade 2 (47.4%) and were in GOLD group D (37.9%). 55.9% of patients were managed at the CS outpatient department. Common comorbidities included hypertension (69.7%), dyslipidemia (54.5%), coronary heart disease (26.9%), and diabetes (24.8%) (Table 1). Common COPD medications included short-acting beta2 agonist plus short-acting muscarinic antagonist (84.8%), inhaled corticosteroid (ICS) plus long-acting beta2 agonist (LABA) (60.0%), long-acting muscarinic antagonist (LAMA) (55.9%), LAMA plus LABA (18.6%) and oral xanthine (44.8%). Rates of influenza and pneumococcal vaccinations were 81.4% and 47.6%, respectively (Table 1).



COPD = chronic obstructive pulmonary disease

Figure 1 Flowchart of COPD patient recruitment to the study.

Table 1 Demographics and baseline characteristics of COPD patients

Characteristics	All patients (n = 145)	Chest specialist (n = 81)	Non-chest specialist (n = 64)	P-value
Age, years	75.0 ± 9.0	75.3 ± 9.6	74.6 ± 8.2	0.569
Male	127 (87.6)	72 (88.9)	55 (85.9)	0.594
Body mass index, kg/m^2	22.6 ± 4.4	22.3 ± 4.2	22.9 ± 4.6	0.226
Smoking, pack-years	30.0 ± 19.9	29.9 ± 20.9	30.2 ± 18.9	0.825
Active smoker	19 (13.1)	11 (57.9)	8 (42.1)	0.848
CAT, points	8.5 ± 6.1	8.8 ± 5.5	8.0 ± 6.7	0.267
mMRC, points	1.7 ± 1.1	1.9 ± 1.1	1.5 ± 1.2	0.038
Spirometry data				
Post-BD FEV_1/FVC, %	57.6 ± 12.9	56.5 ± 12.4	59.0 ± 13.5	0.271
Post-BD FEV_1, %predicted	66.2 ± 24.1	64.5 ± 25.3	68.5 ± 22.3	0.355
BD response	21 (15.9)	10 (12.8)	11(20.4)	0.244
Spirometry grading				0.700
1	35 (26.3)	21 (26.9)	14 (25.5)	
2	63 (47.4)	34 (43.6)	29 (52.7)	
3	25 (18.8)	16 (20.5)	9 (16.4)	
4	10 (7.5)	7 (9.0)	3 (5.5)	
GOLD classification				0.205
A	38 (26.2)	18 (22.2)	20 (31.3)	
B	41 (28.3)	27 (33.3)	14 (21.9)	
C	11 (7.6)	4 (4.9)	7 (10.9)	
D	55 (37.9)	32 (39.5)	23 (35.9)	
Comorbidity				
Hypertension	101 (69.7)	56 (69.1)	45 (70.3)	0.878
Dyslipidemia	79 (54.5)	41 (50.6)	38 (59.4)	0.292
Coronary heart disease	39 (26.9)	29 (35.8)	10 (15.6)	0.007
Diabetes	36 (24.8)	21 (25.9)	15 (23.4)	0.730
Chronic kidney disease	22 (15.2)	17 (21.0)	5 (7.8)	0.028
Cerebrovascular disease	8 (5.5)	5 (6.2)	3 (4.7)	0.697

Table 1 Demographics and baseline characteristics of COPD patients (Cont.)

Characteristics	All patients (n = 145)	Chest specialist (n = 81)	Non-chest specialist (n = 64)	P-value
Medication				
SABA plus SAMA	123 (84.8)	68 (84.0)	55 (85.9)	0.741
ICS plus LABA	87 (60.0)	38 (46.9)	49 (76.6)	<0.001
LAMA	81 (55.9)	44 (54.3)	37 (57.8)	0.674
LAMA plus LABA	27 (18.6)	24 (29.6)	3 (4.7)	<0.001
LABA	3 (2.1)	2 (2.5)	1 (1.6)	0.588
ICS	3 (2.1)	3 (3.7)	0 (0)	0.171
Xanthine	65 (44.8)	38 (46.9)	27 (42.2)	0.570
Procaterol	18 (12.4)	15 (18.5)	3 (4.7)	0.012
Leukotriene receptor antagonist	16 (11.0)	12 (14.8)	4 (6.3)	0.102
Phosphodiesterase-4 inhibitor	9 (6.2)	9 (11.1)	0 (0)	0.004
Macrolide	9 (6.2)	8 (9.9)	1 (1.6)	0.038
Vaccination				
Influenza vaccine within 1 year	118 (81.4)	76 (93.8)	42 (65.6)	<0.001
Pneumococcal vaccine	69 (47.6)	58 (71.6)	11 (17.2)	<0.001

Data presented as n (%) or mean ± SD

BD = bronchodilator, CAT = COPD Assessment Test, COPD = chronic obstructive pulmonary disease, FEV_1 = forced expiratory volume in 1 second, FVC = forced vital capacity, GOLD = Global Initiative for Chronic Obstructive Lung Disease, ICS = inhaled corticosteroid, LABA = long-acting beta2 agonist, LAMA = long-acting muscarinic antagonist, mMRC = modified Medical Research Council, SABA = short-acting beta2 agonist, SAMA = short-acting muscarinic antagonist

When compared to the non-CS group, the CS group had significantly higher proportion of coronary heart disease, chronic kidney disease, and severe symptoms assessed by mMRC (Table 1). Moreover, the CS group had higher prescription rates of LAMA plus LABA, procaterol, phosphodiesterase-4 inhibitor, macrolide, and influenza and pneumococcal vaccinations, but lower prescription rates of ICS plus LABA than the non-CS group (Table 1). In addition, the CS group had significantly higher rates of pulse oximetry measurement than the non-CS group (Table 2).

The overall prevalence of physicians following recommendations was 29.0%. There was no statistically significant difference in these rates between the CS (27.2%) and the non-CS (31.3%) groups (Table 2). Furthermore, clinical outcomes did not differ significantly between the CS and the non-CS groups (Table 2). Also, there were no statistically significant differences in acute exacerbation of COPD between those following and those not following recommendations in both the CS and the non-CS groups (Table 3). The dataset of study participants is shown in S1 File.

Table 2 Comparison in managements and clinical outcomes of COPD patients between the chest-specialist and the non-chest-specialist groups

Variables	Chest specialist (n = 81)	Non-chest specialist (n = 64)	P-value
Disease classification	38 (46.9)	38 (59.4)	0.136
CBC within 1 year	63 (77.8)	55 (85.9)	0.210
CXR within 1 year	78 (96.3)	61 (95.3)	0.768
SpO_2 measurement	77 (95.1)	43 (67.2)	<0.001
Treatment			
COPD education	70 (86.4)	56 (87.5)	0.848
Following recommendations	22 (27.2)	20 (31.3)	0.590
Clinical outcome			
AECOPD with ED visit	28 (34.6)	18 (28.1)	0.408
AECOPD with hospitalization	23 (28.4)	14 (21.9)	0.307

Data presented as n (%)
AECOPD = acute exacerbation of COPD, CBC = complete blood count, COPD = chronic obstructive pulmonary disease, CXR = chest x-ray, ED = emergency department, SpO_2 = oxygen saturation by pulse oximetry

Table 3 Comparison in acute exacerbation of COPD between following and non-following recommendations among the chest-specialist and non-chest-specialist groups

Variables	AECOPD	Following recommendations			P-value
		Yes	No	Total	
Chest specialist	Yes	20 (33.9)	8 (36.4)	28 (34.6)	0.836
	No	39 (66.1)	14 (63.6)	53 (65.4)	
Non-chest specialist	Yes	13 (29.5)	5 (25.0)	18 (28.1)	0.708
	No	31 (70.5)	15 (75.0)	46 (71.9)	

Data presented as n (%)
AECOPD = acute exacerbation of chronic obstructive pulmonary disease

Discussion

This is a study comparing rates of following the international COPD recommendations and clinical outcomes between CS and non-CS in real-world clinical practice. The prevalence was found to be 27.2% in the CS group and 31.3% in the non-CS group (overall rate of 29.0%). These rates were lower than the rates in previous studies of following the GOLD recommendations in real-life clinical practice (misclassifications in 32.8%[4] and inappropriate treatment in 62.1%[12]).

The international COPD recommendations by GOLD[1] suggest that COPD patients should be classified into ABCD groups based on clinical symptoms and risk of exacerbation. Moreover, the recommendations recommend that various treatments depend on disease conditions. However, our results indicated no correlation between clinical outcomes and adherence to the recommendations, and no difference between the CS group and the non-CS group. Nevertheless, rates of vaccinations were significantly higher in the CS group compared to the non-CS group (93.8% vs 65.6% for influenza vaccination and 71.6% vs 17.2% for pneumococcal vaccination). These immunization rates in the CS group were higher than the overall rates in an observational COPD study in Thailand by Saiphoklang N, et al. (71.4% for influenza vaccination and 50.6%for pneumococcal vaccination).[13]

A study of stable COPD in France by Jebrak G, et al.[5] showed that there are discrepancies between COPD recommendations by GOLD and routine treatments. Some treatments such as ICS were overused in mild stages of disease, whereas there was undertreatment by influenza vaccination and pulmonary rehabilitation. An observational study of adherence to COPD recommendations in Turkey by Turan O, et al.[11] demonstrated that 38.4 to 51.8% of COPD patients received unsuitable therapy and 98% of the unsuitable treatment was overtreatment. A study of primary care physicians for COPD management in Greece by Trakada G, et al.[14] showed that 66% of physicians treated patients according to the recommendations and 12.6% prescribed influenza vaccines.

A study on adherence to COPD GOLD recommendations by GPs in a rural area of Italy by Maniscalco M, et al.[15] showed that GPs often diagnosed and empirically treated COPD without confirmative spirometry. Patients in groups A and B were over-treated and 19% of those in group D were under-treated according to GOLD ABCD categorization[15].

A study by Cazzola M, et al.[6] comparing care of COPD patients in Italy to international COPD recommendations found that GPs usually prescribed treatment without the use of spirometry, and/or without assessment of the severity of airway obstruction. Only 31.9% of the patients had had a spirometry test and only 29.9% had visited a specialist. Similarly, a study of spirometry use for COPD management in Hong Kong by Yu WC, et al.[16] showed that only 18.3% of the patients had spirometry done at a diagnostic workup, and only 53.3% had ever had spirometry done. A study in the United States by Salinas GD, et al.[17] demonstrated that GPs' use of spirometry depended on agreement with the recommendations, self-efficacy, perceived outcome expectancy if recommendations were adhered to, and resource availability.[17] Furthermore, adherence to guideline recommendations of long-acting bronchodilator use was predicted by agreement with the recommendations and self-efficacy.[17]

A study on interpretation of pulmonary function tests in asthma and COPD by Raghunath AS et al.[18] showed that agreement in interpretation of the spirometry data between GPs and chest specialists was only 20.4% indicating that interpretation was difficult.

Moreover, a study comparing treatment efficacy between GPs following and not following the COPD recommendations in Italy by Tinelli C, et al.[7] showed that GPs following the recommendations had more outpatient appointments, specialist consultations and higher proportion of classification as severe COPD. However, quality of life and other clinical outcomes including decreased exacerbations, hospitalizations, and medication use were not affected by application of the recommendations[7].

A study of real-life GOLD 2011 implementation among CS in Czech Republic by Koblizek V, et al.[4] found discrepancy between subjective and objective COPD classifications in 32.8% of patients. The most common reason for incorrect classification was incorrect assessment of symptoms. Errors resulted in underestimation in 23.9% and overestimation in 8.9% of patients. The specialists examining 120 patients per month or more were most likely to misclassify their disease (36.7% of all patients). 19.5% of patients received ICS not recommended by the recommendations, and 12.2% of patients were not prescribed ICS which were recommended[4]. Similarly, an observational study of COPD treatment in Italy by Corrado A, et al.[12] showed poor correlation between GOLD international recommendations and real-life clinical practice, resulting in inappropriate treatment in 62.1% of cases. The inappropriateness was due to under-prescription in 7.2% and to over-prescription in 54.9%. COPD exacerbations might have played a role in over-prescription in stages I and II of diseases.[12] A study conducted at a university hospital in northern Thailand by Pothirat C, et al.[19] found that pulmonologists followed national COPD guidelines more closely than internists. The rates and frequencies of severe AECOPD were significantly lower in patients managed by pulmonologists, and the length of hospital stay and cost were significantly lower among the patients with severe AECOPD who required mechanical ventilation. These findings contrast with our study, which might be due to differences in medical policies at each hospital leading to different management and outcomes. Our results found that 36.4% of patients in the CS group, who did not follow the recommendations, had AECOPD, while 25% of patients in the non-CS group had AECOPD. Our results found that 36.4% of patients in the CS group, who did not follow the recommendations, had AECOPD, while 25%

of patients in the non-CS group had AECOPD. Our results found that 36.4% of patients in the CS group, who did not follow the recommendations, had AECOPD, while 25% of patients in the non-CS group had AECOPD. These findings might be attributed to the greater severity of COPD in our patients, as indicated by spirometry grade 3-4 and GOLD class D, in the CS group compared to the non-CS group (9-21% vs. 5-16% and 39% vs. 36%, respectively). Moreover, our study found that physicians in the CS group prescribed more LABA/LAMA, LABA, and PDE4 inhibitors than those in the non-CS group, while physicians in the non-CS group prescribed more ICS/LABA than those in the CS group. These findings might be attributed to the prescribing privileges of physicians in the CS group for LABA/LAMA, LABA, and PDE4 inhibitors, as well as limitations on medical prescriptions among physicians in the non-CS group."

Actually, there are various guideline recom mendations for COPD management other than GOLD such as the European Respiratory Society (ERS) and the British Thoracic Society (BTS). The BTS established the criteria for specialist referral, admission, discharge and follow-up for adults with COPD using appropriate steps tailored to the patient's history and evolving investigations.[20] A large comparative study of real-life COPD medication use in 7 European countries by Rudolf M [21] demonstrated that COPD was both under- and misdiagnosed, there were large differences between different European approaches to drug therapy, and COPD recommendations by the ERS and the BTS were often not followed.[21]

Our study and other studies suggest that there are gaps in COPD management between the CS and non-CS groups especially regarding vaccinations. Therefore, we emphasize the importance of COPD management to improve clinical outcomes.

There are a few limitations of this study. Firstly, this was a prospective cross-sectional study. We collected data on clinical outcomes by reviewing from electronic medical records and asking patients. Therefore, these parameters might lead to misinterpretation of the results. Secondly, this study was conducted in a single research center in Thailand, so the results might not be representative of the whole country. Thirdly, this study was conducted in times of coronavirus 2019 disease (COVID-19) pandemic, which may have influenced the exacerbation rate in COPD patients. Lastly, a small sample size of the population was used in this study. Thus, study outcomes might not be representative of the whole population and some results might not have reached statistically significant differences between groups. A large prospective longitudinal study is required to investigate the correlation between adherence and non-adherence to COPD recommendations in CS and non-CS groups.

Conclusions

One-third of patients were managed following the COPD recommendations in real-world practice. The rates of following the recommendations did not significantly differ between the CS and non-CS groups, nor did the clinical outcomes. Nevertheless, a large prospective study should be conducted to compare clinical outcomes, especially mortality rate, between the two groups.

Financial support

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Compliance with Ethics Requirements

Ethic approval was obtained from the Human Research Ethics Committee of Thammasat University No.1 (Faculty of Medicine), Thailand (IRB No. MTU-EC-IM-0-128/63, COA No. 212/2020). All participants provided written informed consent.

Conflicts of Interest

The authors declare no conflicts of interest.

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Author Contributions

All authors participated in the design of this analysis, data collection and analysis, paper writing, and revision.

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Original Article

Comparison of The Efficacy and Safety of Topical 2% Minoxidil and 5% Minoxidil Solution in Fingernail Growth Rate

Sunatra Nitayavardhana[1*], Khunkheree Rungsriwong[1], Poonkiat Suchonwanit[2], Vanravi Vachatimanont

Abstract

Introduction: Nails are one of the organs which have activity throughout human life. Topical 2% minoxidil has been shown to accelerate nail development by being applied topically on the nails. However, the main mechanism to such effect remains unknown. Moreover, the appropriate preparation and concentration of minoxidil still have not yet been identified. It has been postulated that the higher concentration of minoxidil could accelerate the nail growth more than the 2%, lower concentration.

Objectives: To compare the efficacy and safety of fingernail growth rate between topical 5% minoxidil solution and topical 2% minoxidil solution.

Methods: Thirty participants were randomized into 3 groups: 2% minoxidil, 5% minoxidil, and placebo. Each group of participants was instructed to apply the solution twice daily on the 2nd and 4th finger on both hands. Nail lengths were measured at weeks 4 and 8 by using a digital caliper. Possible side effects were assessed by a dermatologist.

Results: The mean nail growth rate of the 2% minoxidil application (3.32 ± 0.63 mm) was greater than the 5% minoxidil application (2.88 ± 0.50 mm) and placebo (2.87 ± 0.08 mm) at week 8 (*p-value* = 0.002). No cutaneous or systemic side effects were observed.

Conclusions: In conclusion, 2% topical minoxidil has shown to stimulate a greater nail growth rate than the 5% topical minoxidil solution. Therefore, 2% topical minoxidil is an adequate concentration for effectively accelerating nail growth rate, without any side effects.

Keywords: Nail, Nail growth, Minoxidil

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[1] Dermatology Unit, Chulabhorn International Collage of Medicine, Thammasat University, Pathum Thani, Thailand
[2] Dermatology Unit, Ramathibodhi Hospital, Mahidol University, Bangkok, Thailand
*** Corresponding author:** Sunatra Nitayavardhana, Dermatology Unit, Chulabhorn International Collage of Medicine, Thammasat University, Pathum Thani, Thailand, Tel. (+66)081-6619915, Email: sunatra9@gmail.com

Introduction

The nail is a structure that covers the distal part of fingers. It has many functions such as protection of the fingertips, tactile sensation, and thermoregulation via the glomus body (Haneke, 2014). The nail of the middle finger of the dominant hand grows the fastest with the rate of 0.1 mm/day. Whereas, the nail of the little finger grows the slowest. There are many factors that may influence nail growth, such as medications, diseases, and psychological factors. (Geyer, Onumah, Uyttendaele, & Scher, 2004)

In the past, many studies have been conducted to find a drug that can cause the nails to grow faster. However, drugs that affect nail growth in the oral form may have side effects on the body and may interact with drugs that the patient is already taking, so the topical form is used to minimize side effects.

Minoxidil is the medication that has been approved for the treatment of the hair loss.[1] The mechanism for its effect in regrowing hair is not fully understood. It has been postulated that the activated drug affects the ATP-sensitive potassium channels. It opens the potassium channels on the smooth muscle of the arterioles, thus, triggering cell hyperpolarization, which causes vasodilation (Suchonwanit, et al., 2019). In the embryonic stage, the hair follicles and the nail units come from the same origin. Previous research has shown that 2% topical minoxidil can stimulate nail growth (Aiempanakit, et al., 2017). However, it is unknown if increasing the concentration of topical minoxidil would improve the effect of stimulated nail growth. Therefore, in this study, we aimed to evaluate the nail growth rates after applying 2% minoxidil and 5% minoxidil solution.

Methods

Study design

The study was approved by The Human Research Ethics Committee of Thammasat University in accordance with the Declaration of Helsinki, The Belmont Report, CIOMS Guidelines, and the International Practice (ICH-GCP) and registered at clinicaltrials.in.th. The research was a randomized controlled trial. The protocol was conducted from December 2020 to March 2021 at the Dermatology Clinic, Benjakitti park hospital, Bangkok.

Participants

Thirty participants (4 males and 26 females, aged 18-68 years) were enrolled into the study. The inclusion criteria were participants who have normal nails and age range between 17-70 years old. Participants who were pregnant, lactating, individuals with underlying cutaneous or systemic diseases that can affect nail growth, such as thyroid disease and psoriasis, and participants using medications that can affect nail growth, such as retinoids, chemotherapeutic agents, and Itraconazole were excluded. Participants who had history of allergies to minoxidil were also excluded.

The participants were divided into 3 groups by blocked randomization. Each group of participants was instructed to apply 1 drop (0.05 ml) of the solution (placebo, 2% minoxidil, or 5% minoxidil) to the index and ring finger of both hands on the proximal nail fold. The thumb, middle and little fingernails were not assessed, because they grow at a different rate in comparison with the index and ring fingernails. Solution was applied by using cotton wool and rubbing until the solution was completely absorbed and then, avoiding hand washing for at least 30 minutes afterward. The dosage was approximately 20 milligrams/ml for 2% minoxidil and 50 milligrams/ml for 5% minoxidil. The nail length was measured from the deepest edge of the marked line to the proximal nail fold by using the digital caliper (Mitutoyo, Kawasaki, Japan).

A blinded dermatologist measured the nail length twice and then computed the average. The participants' fingernails were photographed and marked at the baseline. Moreover, examination of blood pressure, pulse rate and cutaneous side effects were recorded. Figure 1 shows the research protocol.

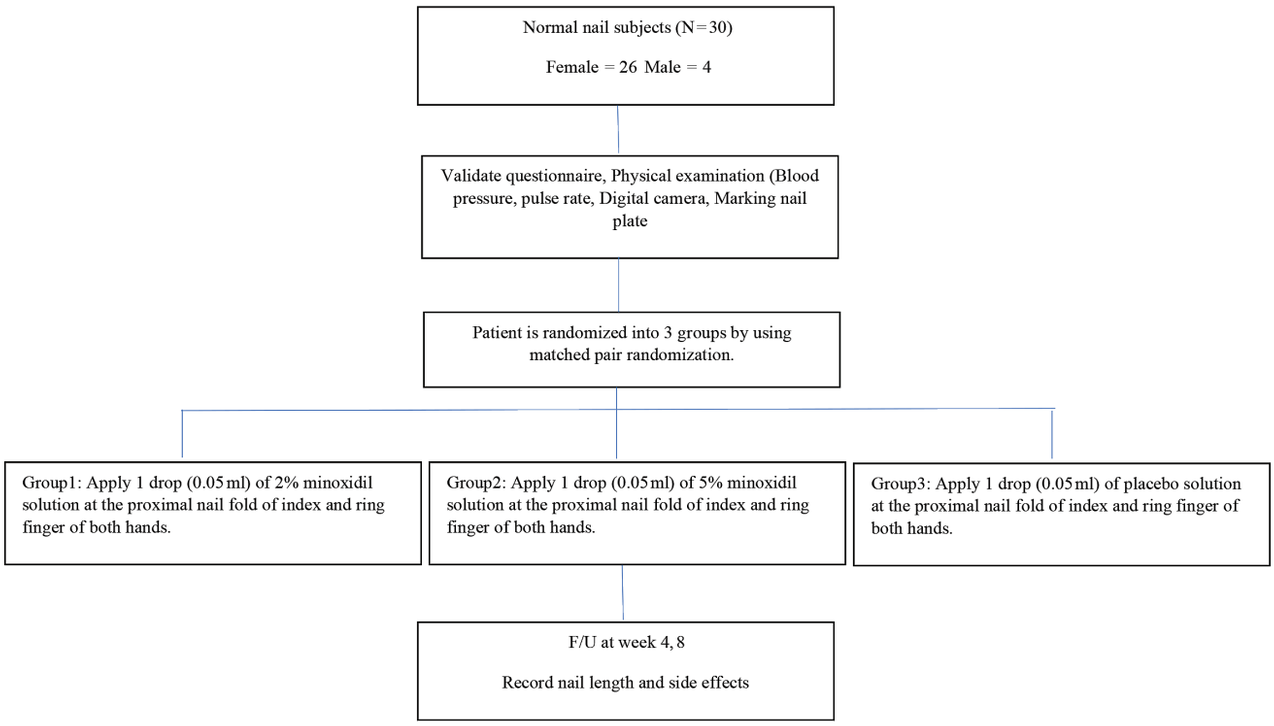

Figure 1 shows the research protocol

Outcomes

The primary outcome was to compare the rate of nail growth between 2% and 5% concentrations of minoxidil for 8 weeks. The secondary outcome was to observe for possible adverse reactions.

Statistical analysis

Statistical analyses were calculated by using Stata version 14.0 (College Station, TX, USA). The sample size was calculated using a sample size for a two-sample means test formula. Quantitative variables were explained by using the mean and standard deviation for continuous variables and number and percentage for categorical variables. Comparison of the mean nail growth rate between the 2% minoxidil, 5% minoxidil, and placebo were performed by using one-way ANOVA, Kruskal-Wallis independent T-test, and Mann-Whitney U test. A *P-value* < 0.05 was defined as statistically significant. Moreover, the paired t-tests were used to compare other continuous variables before and after treatment.

Results

Participants

A total of 30 participants were enrolled into the study. Twenty-six participants (86%) were female. Eighty-two percent of participants were right-hand dominant. The mean age was 41 years. The underlying diseases of the participant were hypertension, dyslipidemia, and gout. Four participants in the placebo group used calcium, vitamin D, and vitamin C. The demographic data in the 2 different groups were not statistically significant. The demographic data is shown in Table 1.

Table 1 Demographic data

	Placebo	2% minoxidil	5%minoxidil	P-value
Age	42.10 + 15.87	47.80 + 17.15	35.80 + 16.80	0.288
Sex				
Male	0.(0.00)	2(20.00)	2(20.00)	0.507
Female	10(100)	8(80.00)	8(80.00)	
Drug/Supplement				0.668
Yes	4(40.00)	2(20.00)	2(20.00)	0.507
No	6(100.00)	8(80.00)	8(80.00)	
Handedness				1.00
Right	9(90.00)	8(80.00)	8(80.00)	
Left	1(10.00)	2(20.00)	2(20.00)	

Nail growth

Before starting the protocol, we marked the measure point on 2nd and 4th fingers on both hands. The measurement of nail length was done at baseline, week 4 and week 8.

The rates of nail growth were compared between the three groups: 2% minoxidil, 5% minoxidil, and placebo group. At four weeks, the mean nail growth rate was 3.58 ± 0.66 mm/month, 3.48 ± 0.65 mm/month, 3.15 ± 0.75 mm/month in 2% minoxidil, 5% minoxidil, and placebo, respectively. The length of nail growth in the 2% minoxidil group was higher than the placebo group with statistical significance (P = 0.019). However, there was no difference between 2% minoxidil and 5% minoxidil. Furthermore, at eight weeks, the mean of nail growth rate in 2% minoxidil group was significantly higher than both placebo and 5% minoxidil (P = <0.001). The average nail growth rate at week 8 of the 2% minoxidil group was 3.32 ± 0.63 mm/month, exceeding other groups, 2.88 ± 0.50 mm/month in the 5% minoxidil group, and 2.87 ± 0.54 mm/month in the placebo group. There was no statistically significant difference between the placebo and 5% minoxidil group. As a result, 2% minoxidil solution has shown to be an adequate concentration to effectively stimulate the nail growth after 4 and 8 weeks of topical application. The mean nail growth rates are shown in Table 2.

No adverse reactions observed during the study in all groups.

Table 2 Comparison of the means of nail growth rate between 2% minoxidil, 5% minoxidil and placebo.

	Placebo (N = 40)	2%minoxidil (N = 40)	5%Minoxidil (N = 40)	P-value
The mean nail growth rate at week 4	3.15 + 0.75	3.58 + 0.66	3.48 + 0.65	0.017
The mean nail growth rate at week 8	2.87 + 0.54	3.32 + 0.63	2.88 + 0.50	<0.001

One-way ANOVA, multiple comparison: Scheffe

Discussion

Currently, topical medications that can stimulate nail growth is understudied. This study showed an increase in nail growth rate on all fingernails after applying topical minoxidil. Both concentrations, 2% and 5% topical minoxidil solutions could stimulate nail growth compared to the placebo, with statistical significance at week 4. In contrast, only 2% minoxidil solution could stimulate nail growth with statistically significant difference from the placebo at week 8. There were no local or systemic side effects observed throughout the study.

We compared the result with the previous literature. Aiempanakit found that 5% minoxidil could stimulate nail growth in the first month (Aiempanakit et al., 2017). The same result was obtained from our research, that 5% minoxidil has a higher nail growth rate than the placebo with statistical significance only in the first 4 weeks.

The mechanism of minoxidil in promoting hair growth remains unknown. It was found that topical minoxidil could upregulate VEGF (Yano, Brown, & Detmar, 2001) or nitric oxide (Yum et al., 2018). It could also activate ATP-sensitive potassium (KATP) channels and relax vascular smooth muscle (Farrokhi, Gashti, Hoormand, Bakhtiarian, & Habibi, 2019), decrease blood pressure, and increase blood flow (Gümüş, Ödemiş, Yılmaz, & Tuncer, 2012). It could be possible that topical minoxidil can accelerate nail growth by increasing cutaneous blood flow to the nail.

With regard to the question of why greater growth rates in 5% minoxidil and 2% minoxidil were detected only in the first month, it could possibly be an autoregulation of nail blood flow in response to different concentrations of minoxidil and with higher concentrations there might have been earlier adaptation of the blood circulation in the hand. Another opinion that may support our result is related to prostaglandin (PGE2) from the minoxidil solution. It was discovered that Minoxidil can increase PGE2 by activating prostaglandin endoperoxide synthase-1 enzyme. (30) PGE2 upregulates genes in the β-catenin pathway of dermal papilla cells. The Dermal papilla are found in the nail matrix at the proximal nail fold. Moreover, wnt-β-catenin signaling is also found at the nail stem cell, which is used in nail plate production. This study could explain the limited cell response of stem cells in the proximal nail fold.

Topical minoxidil is a well-known medication that is commonly used to treat alopecia. Using topical application, instead of oral ingestion, helps to eliminate systemic side effects that could occur such as generalized hair growth. Moreover, the nails and hair follicles similarly share some common structures. Thus, it is possible that minoxidil could possibly increase nail growth just like it increases hair growth. This study has shown that topical minoxidil can effectively stimulate nail growth, even though the effect of minoxidil on nail growth might not be as substantial as stimulating hair growth. This difference in stimulating growth is understandable, as the absorption through the thick keratinized nail is much more difficult than in the scalp or mucosal area. In addition, further studies could be extended to investigate better routes of drug delivery and increased drug absorption. Also, larger patient samples and longer treatment periods could be considered, to identify the mechanism of action of minoxidil in nail growth stimulation. Our study was limited by its small sample size and 2 months application period. Therefore, we recommend larger trials with longer periods of application to see the trend of treatment results.

In conclusion, topical application of both 2% and 5% minoxidil to proximal folds can increase the nail growth. 2% minoxidil can significantly increase the nail growth at both 4 weeks and 8 weeks. Thus, 2% concentration is adequate to stimulate nail growth without inducing any adverse side effects. Hopefully, this study could be useful for patients who have nail disorders and assist them to regain their nails back faster.

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Original Article

Detection of Circulating Tumor Cells in Peripheral Blood by Use of The High-Gradient Magnetic Separation Technique in Ovarian Tumor Patients

Chamnan Tanprasertkul[1,2 *], Charintip Somprasit[1], Komsun Suwannarurk[1], Nipattha Vinayanuvattikhun[1], Prapat Suriyaphol[3], Sebastian Chakrit Bhakdi[4]

Abstract

Objective: To evaluate the performance of the high-gradient magnetic separation (HGMS) technique in detecting circulating tumor cells (CTCs) for the preoperative diagnosis of ovarian cancer.

Methods: Women who had ovarian tumors and were admitted to Thammasat University Hospital during January 2018-December 2019 were enrolled into the study. Ten milliliters of fresh peripheral blood for HGMS were collected within 24 hrs prior to surgery. After healthy cell depletion by HGMS, the remaining cells including CTCs were spun onto gelatinized standard laboratory slides and stained with a panel of specific antibodies against CD45, CD31, CD34, CD73, CAM5.2, C-11, VIM and PKM2. The findings were classified into five classes, as based on cell types and their quantities: Classes I-III were categorized as a negative test and Classes IV-V were categorized as a positive test. The CTCs findings were compared to the final histopathological report.

Results: There were 67 participants in the study, with a mean age of 44.8 years. The detection rate of the test was 72.92%. Overall sensitivity and specificity were 45.45% and 94.12%, respectively. The accuracy of this method was 85.48%, with a negative predictive value of 88.89% and a positive predictive value of 62.50%.

Conclusion: The HGMS technique has a promising capacity for detecting ovarian cancer CTCs in patients with ovarian tumors. This technique should be optimized further and utilized, instead of a tumor markers, as a preoperative method for detecting ovarian cancer in the near future.

Keywords: circulating tumor cells, ovarian cancer, peripheral blood, high-gradient magnetic separation

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[1] Department of Obstetrics and Gynecology, Faculty of Medicine, Thammasat University, Thailand
[2] Advanced clinical innovative research in obstetrics and gynecology group, Thammasat University, Thailand, 12120
[3] Division of Bioinformatics and Data Management for Research, Research Group and Research Network Division, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand ,10400
[4] Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand; X-ZELL, 133 Cecil Street, #06-02 Keck Seng Tower, Singapore 069535, Singapore
***Corresponding author:** Tanprasertkul C., Department of Obstetrics and Gynecology, Faculty of Medicine, Thammasat University, Pathum Thani, Thailand 12120; Tel. 02-9269343; E-mail: chamnandoctor@gmail.com

Introduction

Ovarian cancer is a common gynecologic cancer, not only in Thailand, but globally. It has a high mortality rate because there are no obvious symptoms[1] and consequently, the delayed diagnosis often leads to a substantially higher mortality rate. In the preoperative assessment of ovarian tumors, gynecologists may fail to accurately diagnose ovarian cancer due to the limited precision of existing diagnostic tools.

The current screening method uses serum tumor markers like CA 125 and HE4 in peripheral blood, along with trans-vaginal ultrasonography. The results from the use of these investigational tools still have limitations in terms of accuracy. Moreover, this method has a high false-positive rate and lacks value in health economics.[2]

With the development of biotechnology, previous studies have found that circulating tumor cells (CTCs) in cancer patients can be detected from an early to an advanced stage of the disease. This detection of cancer cells in the bloodstream may be used to support the diagnosis and assist the decisions of gynecologists in planning management, especially for surgical procedures. In addition, CTCs could be used to monitor the stage of the disease or inform the clinician of its prognosis.[3,4,5] However, there are still no conclusive methods for detecting CTCs.

The measurement for the presence of cancer cells in the bloodstream is highly complex and difficult, especially because the number of such cells is so very small. Currently, the method of searching for cancer cells in the bloodstream is to capture and pull cancer cells directly from the bloodstream. Most research relies on the use of immunological methods, for example, an antibody to the epithelial cell adhesion molecule (EpCAM).

The challenge associated with the aforementioned method lies in the significant heterogeneity of cancer cells possibly present in the bloodstream, particularly in terms of the variety of surface markers. As a result, the direct trapping approach for identifying circulating tumor cells (CTCs) frequently fails to capture cells that exhibit divergent properties. Moreover, studies for detecting CTCs found that even with the same patient's blood samples, different methods produced different results.[6,7,8]

This study aims to evaluate the high-gradient magnetic separation (HGMS) technique, which is a newly developed method for searching for cancer cells in the bloodstream, or CTCs, in the preoperative prediction of ovarian cancer.

Methods

The study is based on a diagnostic design with prospective data collection. It was conducted at the Department of Obstetrics and Gynecology of Thammasat University Hospital, Thailand, during January 2018 - December 2019 following approval from the ethical institute committee.

We enrolled women with ovarian tumors who had to undergo ovarian surgery, using either laparoscopy or laparotomy. They were 18 - 65 years old, completely understood the process of this study and had given their written informed consent to the study. Any with suspected or confirmed other organ cancers of other types were excluded from the study. The sample size was calculated by the formula $n = \frac{Z^2 p\,(1 - p)}{d^2}$. The proportion (p) of ovarian cancer in our institution was 0.20. We used an error (d) = 0.10 and alpha (α) = 0.05. The participant number was then set at 62. We compensated for a data loss of 15%, and the final total needed sample size was set at 72.

An additional 10 mL of blood was drawn from each patient on the day of hospitalization prior to surgery. This blood sample was collected into a container pre-filled with either EDTA or heparin by qualified nursing staff or specialized laboratory personnel. All specimens were accurately labeled in accordance with the trial protocol, including pertinent information about the participating patients. Clinical data were meticulously recorded in case report forms. On the same day as collection, the blood samples were stored at a temperature of 4°C and promptly transported to the Laboratory of Bioinformatics and Research Data Management Unit within the Research Center at the Faculty of Medicine, Siriraj Hospital, Mahidol University. Cellular analyses were conducted on the same day, and investigators responsible for these analyses were blinded to both the operative outcomes and the pathological findings.

Laboratory Technique

All samples were processed as follows. The blood samples of the participants were subjected to hemolysis by using of a lysing buffer solution. The blood was then washed with a phosphate buffer saline (PBS) and mixed with paramagnetic nano-beads for 10-15 min. These nano-beads bound to white blood cells without binding to cancer cells. The cell suspension was then placed in a column so that the normal blood cells that were magnetically labeled became fixed in the column. The cancer cells in the blood passed out of the column and were stored for further HGMS separation. Cells were spun onto gelatinized standard laboratory slides and stained with a panel of specific antibodies against CD45, CD31, CD34, CD73, CAM5.2, C-11, VIM and PKM2.

The slides were examined under a fluorescence microscope. The findings were classified into five classes, as based on cell types and their quantities: negative for malignancy (class I), atypical cells found but negative for malignancy (class II), suspicious for malignant cells (class III), strongly suggestive for malignant cells (class IV) and conclusive for malignant cells (class V). The CTCs Cytopathological Criteria were classified as the followings:

I	• No CD45- cells
II	• CD45- cells without positive markers (CEC/CTC vs plasma cells) • Less than five giant polyploidic cells: CD31+CD34=CD73-VIM=CK-PKM2-CD45- (Megakaryocyte lineage) • Cells with aneuploidy: CD31+CD34-CD73-VIM-CK-PKM2-CD45-
III	• Less than five single cells: CD31=CD34+CD73-VIM+CK-PKM2-CD45- (angiogenic tip cell: tumor-derived vs inflammatory) • Five or more giant polyploidic cells: CD31+CD34=CD73-VIM=CK-PKM2-CD45- (Megakaryocyte lineage) • More than one large cell: CD31-CD34=CD73-VIM+CK-PKM2-CD45- (mesenchymal CTC vs hematopoietic stem cell) • Both conditions with or without aneuploidy
IV	• Binucleated cells: CD45- • Less than five single cells: CD31-CD34-CD73-VIM=CK+PKM2=CD45-* • One clump: CD31=CD34+CD73=VIM=CK-PKM2=CD45-** • One or more large cells with aneuploidy: CD45- • More than one cells with pronounced aneuploidy: CD31=CD34+CD73-VIM+CK=PKM2=CD45- • More than five single cells: CD31=CD34+CD73-VIM+CK-PKM2=CD45- • One or more single cell: CD31=CD34+CD73+VIM=CK=PKM2=CD45-
V	• One clump: CD31=CD34=CD73=VIM=CK+PKM2=CD45-** • More than one clump: CD31=CD34+CD73=VIM+CK=PKM2=CD45- • More than five CD31-CD34-CD73-VIM=CK+PKM2=CD45- cells* • One or more CD45- cell in atypical mitosis*** • One or more aneuploidic cell: CD31=CD34+CD73+VIM+CK=CD45- • One or more cell with or without aneuploidy: CD31=CD34+CD73+VIM=CK=CD45-

The CTCs findings were compared to the final histopathological reports. All the interpreters were blinded to the concluding results. Classes I-III were categorized as a negative test and Classes IV-V were categorized as a positive test.

Results

Sixty-seven participants, with a mean age of 44.8 years, were included. 62 cases had the complete data for analysis. From the pathological test results, 82.26% of the cases were not found to have ovarian cancer. Most of these were diagnosed with endometrioma and benign cystic teratoma. Of the remaining patients, 11 out of 62, or 17.74%, were confirmed to have ovarian cancer. The majority of these were characterized by the presence of epithelial cells, including types such as serous cystadenocarcinoma, clear cells, and endometrioid cells.

Table 1 presents a classification of circulating tumor cells (CTCs) for the purpose of comparing non-ovarian cancer patients with ovarian cancer patients. In non-cancer patients, most of the detection of CTCs, as much as 94.11%, was found in classes I to III, a true negative. Specifically, there were CTCs detections of 76.47%, 5.88% and 11.76% of patients in classes I, II, and III, respectively. Only 3 out of 62, or 4.83%, were found with CTCs in class IV, which was a false positive. None of the benign ovarian tumor patients were found in class V. In the patients with ovarian cancer, CTCs were detected at 45.45% (true positive) with 9.09% and 36.36% in classes IV and V, respectively. The false negatives in CTC classes I, II & III were 18.18%, 9.09% & 27.27%, respectively, totaling 54.54 %. The ROC curve of detection of CTCs was shown in Figure 1, in which the detection rate of the test was 72.92 %. Overall sensitivity and specificity were 45.45% and 94.12%, respectively. The accuracy of this method was 85.48%, with a negative predictive value of 88.89% and a positive predictive value of 62.50%.

Table 1 Comparison of CTCs occurrences between non-ovarian cancer and ovarian cancer patients

CTCs Class	Non-ovarian cancer		Ovarian cancer	
	No.	%	No.	%
I	39	76.47	2	18.18
II	3	5.88	1	9.09
III	6	11.76	3	27.27
IV	3	5.88	1	9.09
V	0	0.00	4	36.36
Total	51	100.00	11	100.00

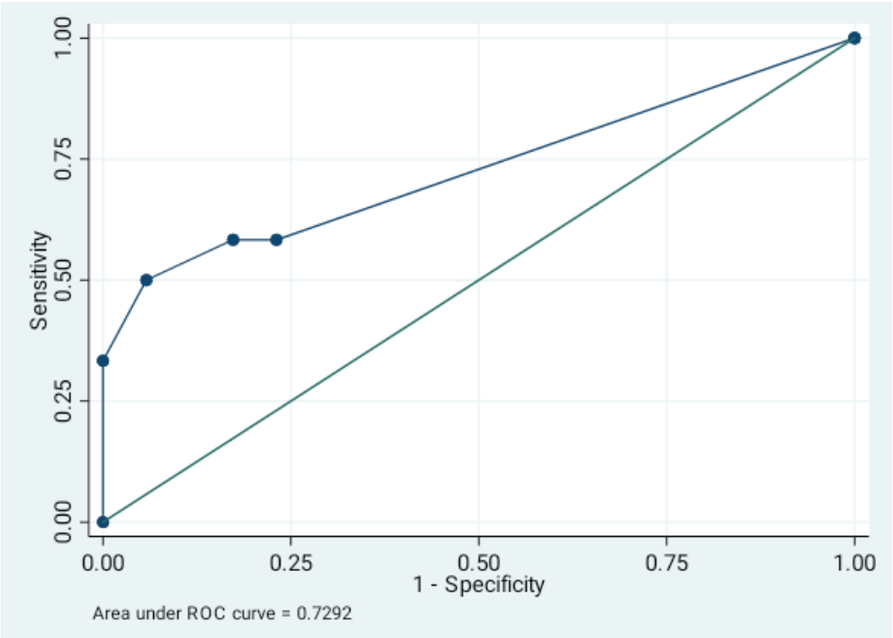

Figure 1 Graphical plot showing diagnostic performances of high-gradient magnetic separation (HGMS) technique as the area under the curve of detection of circulating tumor cells (CTCs)

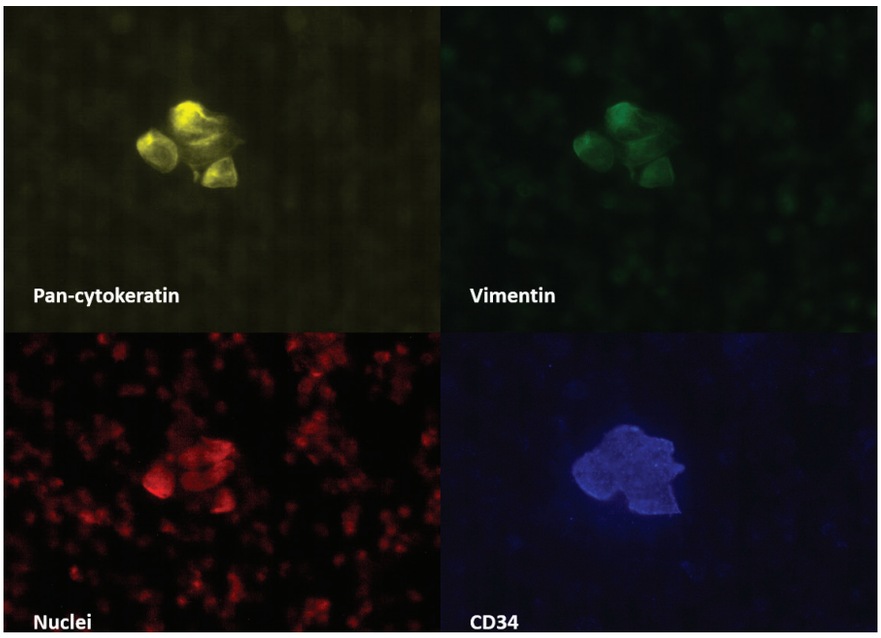

Figure 2 Image of a pan-cytokeratin positive ovarian cancer cell clump, or giant polyploidic cell, in peripheral blood detected and examined under a fluorescence microscope. Note the cytosolic localization of cytokeratin and vimentin as opposed to the pan-membrane staining of CD34, homogenously covering the complete surface of the clump. The nuclear view shows smaller white blood cell nuclei surrounding the four largest, strikingly anisokaryotic cancer cell nuclei. Fluorophores: pan-cytokeratin PE, vimentin AlexaFluor488, CD34 Brilliant Violet 421, nuclei Draq5.

Discussion

Our study results demonstrate the ability of the HGMS technique to detect CTCs in the blood of ovarian tumor patients. The percentage of patients in the ovarian cancer group with true-positive diagnoses was 45.45%, which means that almost half of the preoperative ovarian cancer cases would be well prepared with a specialist team and gynecologic oncologist. Also, this test had an accuracy of 72.92% with a confidence interval between 55.32% to 90.52% (Figure 1), which was sufficient to differentiate benign tumors from malignancies. This outcome is consistent with previous reports[9, 10], which found CTCs in ovarian cancer patients, yet those reports differed from our current study, because prior research was conducted in confirmed ovarian cancer cases, while our study consisted only of suspected or newly identified cases. In our study, we utilized a mixed population comprising both benign and malignant ovarian tumors, which resulted in a low prevalence rate of ovarian cancers. Additionally, the study encompassed a diverse range of cell types and cancer stages. As a consequence, the performance of detection was not particularly high. With statistical calculation, the sensitivity and specificity of this test were found to be 45.45% and 94.12%, respectively. The negative predictive and positive predictive values were 88.89% and 62.50%, respectively. These predictive values could serve as a forecast of non-cancer cases with high predictive performance. HGMS is the cell isolation technique for the separation of large numbers of cells, as based on specific cell-surface markers. The present study adopted high-gradient magnetic separation for the removal of white blood cells, or in other words, it applied a negative rare-cell isolation approach. Use of this technique was followed by highly multiplexed immunostaining on standard laboratory slides. The combination of these two techniques allowed us to isolate tumor-associated cells in a manner independent of the expression of a single antigen and to analyze epithelial, mesenchymal and endothelial antigens on the single-cell level simultaneously (Figure 2). Applying classical cytopathological criteria of malignancy to cells characterized in this way seems to show promise as an adjunct tool in the diagnostics of ovarian cancer.[11, 12]

There were several limitations in this study. The finding of CTCs did not show an association with ovarian cell types, stages of malignancy or serum tumor markers such as CA 125 or HE4, because of the small sample sizes and the variety of ovarian cancer cell types. Moreover, the likelihood of a positive diagnosis of cancer was rather low, possibly because our objective was based on a strategy targeting newly diagnosed ovarian cancer cases.

Conclusion

The HGMS technique was utilized for the pre-operative detection of CTCs in newly diagnosed ovarian cancer patients. The accuracy of the technique was satisfactory, and this method has a promising capacity. Optimizing the HGMS technique might allow it to be utilized, instead of tumor markers, as a pre-operative method for detecting ovarian cancer.

Conflict of interest

The authors declare that there is no conflict of interest

Acknowledgement

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Original Article

Effect of *Ocimum americanum* Water Leaf Extract on Antioxidant System in Rat

Pornrut Rabintossaporn[1*], Nuntiya Somparn[2], Amornnat Thuppia[1], Suphaket Saenthaweesuk[1]

Abstract

Introduction: *O.americanum* has been reported to possess various medicinal properties. No study has been conducted in the antioxidant properties of *O.americanum* from the Thailand region.

Objectives: The objective of this study was to examine the antioxidant and cytoprotective aspects of the herb *O.americanum.*

Methods: Aqueous leaf extract of *O.americanum* was screened for phytochemical contents. Antioxidant properties were determined by the DPPH method. 24 male Sprague-Dawley rats were fed aqueous leaf extract for 28 days. Blood was drawn and collected to estimate the levels of BUN, creatinine, AST, ALT, and ALP and then the rats were sacrificed. Their liver tissues were collected and processed for histological study. Western blot analysis was performed to determine the expression of γ-GCL.

Results: Phytochemical contents of *O.americanum* are phenolic compounds, alkaloids, cardiac glycosides, tannin, and steroids. The phenolic content was 41.27 ± 1.86 mg/gram of gallic acid equivalents. DPPH assay of the free radical scavenging activity determined that IC_{50} was 36.91 ± 0.66 µg/ml (compared to BHT = 12.34 ± 1.14 µg/ml). Throughout the 28 days of the experiment, the rats did not seem to be adversely affected by the extract and were found to have normal ranges of serum AST, ALT, ALP, BUN, and creatinine. The histology of their liver tissue was normal. The protein expression of γ-GCL, which is a key enzyme in GSH biosynthesis, was found to have increased significantly compared with normal controls.

Conclusions: The study found that *Ocimum americanum* extract does have antioxidative properties and can induce cytoprotective properties *in vivo*.

Keywords: Antioxidative properties, DPPH, γ-GCL, Liver histology, *Ocimum americanum*

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[1] Division of Anatomy, Department of Preclinical Science, Faculty of Medicine, Thammasat University, Pathum Thani, Thailand
[2] Division of Pharmacology, Department of Preclinical Science, Faculty of Medicine, Thammasat University, Pathum Thani, Thailand
*** Corresponding author:** Pornrut Rabintossaporn, Division of Anatomy, Department of Preclinical Science, Faculty of Medicine, Thammasat University, Pathum Thani, Thailand, Email: pornrutrabintossaporn@gmail.com, Tel +66 291269710

Introduction

Different forms of free radicals are continuously generated at low levels in cells to regulate several physiological functions and are eliminated by an antioxidative system in the body. An imbalance between free radical production and elimination results in oxidative stress[1]. Enhanced antioxidant activity can reduce the oxidative damage from free radicals, and thus can decrease the risk of developing many free radical-induced illnesses,[2] such as malignancy, autoimmune, cardiovascular, and neurodegenerative diseases.[3] Several plants have been reported to produce antioxidant activity from their phytochemical contents: such as flavonoid, tannin, benzyl-isothiocyanate, and other phenolic compounds.[4,5]

There are many methods to assess the antioxidative capacity of a compound. Among them, 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is one of the widely used methods. It is based on a free radical scavenging property of an antioxidative substance in which the DPPH radical, with an absorption of 515-525 nm, is converted into a colorless compound after reduction by the antioxidant.[6,7]

Gamma glutamylcysteinylglycine (GSH) is the crucial endogenous antioxidant compound involved in cellular redox homeostasis[8] and the glutathione redox system is the body's first-line defense against oxidative stress.[9] GSH is synthesized in the cytosol. The rate-limiting step of GSH biosynthesis requires the cytosolic enzyme gamma glutamate-cysteine ligase (γ-GCL) for glutamylcysteine formation[10] and then glutathione synthetase to link glycine to glutamylcysteine to form GSH.[11]

The genus *Ocimum* which belongs to the family Lamiaceae, has been widely used as a dietary plant. In Thailand, there are 5 species in this genus which are *O.americanum* L., *O.basilicum* L., *O.gratissimum*, *O.tenuiflorum* L. and *O.kilimandscharicum* Baker ex Gurke.[12] There is scientific research to support the health benefits of some of these species. Among them is *Ocimum americanum.*

O.americanum, commonly known as hoary basil has been reported to possess various medicinal properties. Its seed has been used to treat headaches, nausea, vomiting, constipation[13] and fungal infection.[14] Its seed extract could inhibit bacterial growth such as *Salmonella* spp, *Escherichia coli* O157:H, *Campylobacter jejuni* and *Clostridium perfringens*.[15] Water extract from Indian *O.americanum* has been reported to possess hypoglycemic and antioxidant effects.[16] Moreover, previous phytochemical studies suggested that *O.americanum* contains flavonoids, glycosides, terpenoids, tannins, phenols, saponins and steroids.[17, 18] The objective of this study was to examine the antioxidant and cytoprotective aspects of the herb *O.americanum.*

Methods

2.1 Plant material identification

The plant was collected from Pathum Thani province, Thailand in January 2016. The botany identification was performed by the Department of Pharmacognosy and pharmaceutical sciences, Prince of Songkla University (Herbarium Specimen SKP 095 15 01 01).

2.2 Preparation of the extract

2,500 g of air-dried leaves of *O. americanum* were chopped finely, added 1 ratio of hot water to the chopped leaves, boiled in a stainless boiler for 2 hours (100 ºC), and then filtered with cheesecloth. The clear filtrate was collected and freeze-dried (-80 ºC) and evaporate with FTS system evaporator model Dura Dry (Science Engineer International CO., LTD., USA). The weight of the water extract was recorded. The percentage yield was 10.40%.[19]

2.3 Preliminary phytochemical testing

The extract was screened for its phytochemical contents by the standard method according to Thai Herbal Pharmacopoeia Vol. I, 1998.

2.4 Estimation of total phenolic content

Total phenolics were determined by a spectrophotometric method. The absorbance of the solution was recorded at 725 nm. A standard curve of absorbance versus concentration of gallic acid was plotted. The percentage of total phenolic content was calculated and expressed as % gallic acid.[20]

2.5 DPPH assay

Free radical scavenging capacity of the water extract of *Ocimum americanum* (OA) was tested by a modified DPPH assay in three in vitro models using butylated hydroxytoluene (BHT) as a

standard.[6] In brief, 100 µL of DPPH solution and the same volume of diluted OA extract were mixed in a 96-well microplate and held at room temperature for 30 min. BHT was used as a positive control. The absorbance was measured at 520 nm with a blank containing DPPH. The DPPH radical scavenging activity was calculated and expressed as IC50.

2.6 Western blot analysis of γ-GCL protein expression

Western blot analysis was used to determine the expression of γ-GCL and β-actin in liver tissue. RIPA buffer (#9806, Cell Signaling Technology, Inc. MA, USA) was used to lyse tissues with addition of protease inhibitor cocktail (M221: Amresco, OH, USA).

The protein samples were mixed with SDS loading buffer and subjected to separation by electrophoresis in 10% SDS polyacrylamide gel.

The separated proteins are transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked using 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST buffer). After blocking for one hour at room temperature and rinsing by TBST, the membrane was overnight incubated with the primary antibody in TBS. After washing with TBST, the membrane was incubated with the horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for one hour.

After rinsing the membrane to remove unbound antibody by TBST buffer, the membrane was incubated in enhanced chemiluminescence (ECL) substrate solution (Super Signal West Pico Chemiluminescent Substrate: ThermoScientific, IL, USA). Densities of each protein band were visualized and analyzed with the aid of Image Studio version 2.1 software (Odyssey Imaging System LT-COR® biosciences, USA).

2.7 Experimental animals and designs

Twenty-four adult male Sprague-Dawley rats (200-250 gm) were used and ethical committee clearance was obtained from Institutional care and use committee of Thammasat University (IACUC No. 009/2558). Experiments were conducted under Animals for Scientific Purposes Act, B.E. 2558 (A.D. 2015), Government of Thailand. All institutional and national rules for the care and utilization of laboratory animals were followed.

All animals were held in standard laboratory conditions for one week. The conditions were temperature-controlled environment (25 ± 1ºC), 55 ± 5% relative humidity, regular 12 hours light/12 hours dark cycles. They were fed with standard rat chow diet and water ad libitum.

The animals were divided into four groups (n = 6)

Group I: Control group (given distilled water)
Group II: OA extract at the dose of 100 mg/kg BW/day
Group III: OA extract at the dose of 200 mg/kg BW/day
Group IV: OA extract at the dose of 400 mg/kg BW/day

The rats received the extract according to their group for twenty-eight days.

2.8 Biochemical analysis

Blood was drawn from the experimental animals on the twenty-eighth day after they received the extract. The serum was collected to estimate the levels of blood urea nitrogen (BUN), creatinine (Cr), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) by the laboratory unit at Thammasat University Hospital.

2.9 Liver histology study

After twenty-eight days of the study, the rats were anesthetized and sacrificed. Their liver tissues were collected and fixed in 10% formalin for one week, and then embedded in paraffin. The paraffin blocks were cut into 5 µm thick serial sections, then stained with hematoxylin-eosin (H&E) and examined for histological study by photomicroscope.

2.10 Statistical analysis

Data were expressed as mean ± S.E. All statistical analyses were performed by one-way analysis of variance (ANOVA). $P < 0.05$ was considered significant.

Results

3.1 Phytochemical screening and total phenolic compound

The phytochemical screening of OA leaf extract revealed the presence of alkaloid, cardiac glycosides, tannin, steroid, and phenolic compounds. The phenolic content in OA was found to be 41.27 ± 1.86 mg of gallic acid equivalents per gram.

3.2 Antioxidant activity studies

Free radical scavenging activity assessed by DPPH assay gave IC_{50} of 36.91 ± 0.66 μg/mL (that of BHT was 12.34 ± 1.14 μg/mL). The protein expression of antioxidant enzymes, γ-glutamylcysteine ligase (γ-GCL) in the liver is shown in Figure 1.

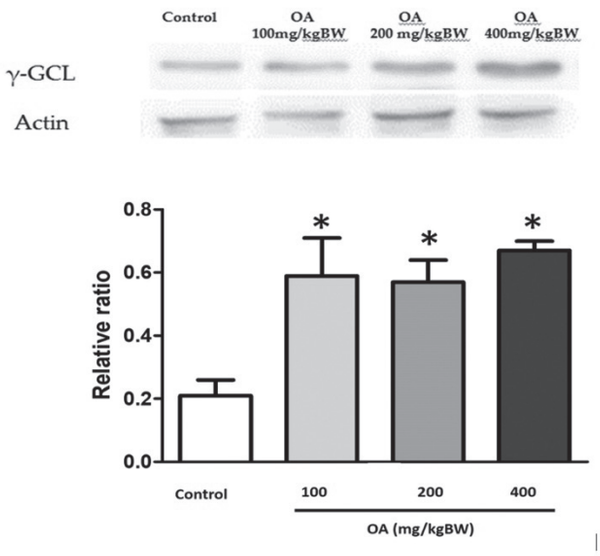

Figure 1 The protein expression of γ-GCL in the liver tissue of the control and OA treated rats. *p < 0.05.

3.3 Biochemical analysis

Biochemical parameters: AST, ALT, ALP, BUN, and Cr are shown in Table 1.

Table 1 Serum biochemical parameters of the control and OA treated rats

	AST (U/L)	ALT (U/L)	ALP (U/L)	BUN (mg/dL)	Cr (mg/dL)
Control	120 ± 5.51	43.33 ± 1.45	80.33 ± 5.93	20 ± 1.16	0.37 ± 0.03
OA 100 mg/kg BW	84.67 ± 7.22	36.67 ± 2.67	68.67 ± 2.40	19 ± 2.00	0.23 ± 0.03
OA 200 mg/kg BW	81.33 ± 6.17	31.67 ± 1.86	64.33 ± 4.37	16.33 ± 0.33	0.20 ± 0.00
OA 400 mg/kg BW	83 ± 6.43	37.67 ± 1.67	69.67 ± 2.19	16.67 ± 0.88	0.27 ± 0.03

Note: AST; Serum aspartate aminotransferase, ALT; alanine aminotransferase, ALP; alkaline phosphatase, BUN; blood urea nitrogen and Cr; creatinine

3.4 Liver histology study

As shown in Figure 2, there is no change of normal hepatic lobular pattern, portal triads, central vein; hepatocellular trabeculae are regularly distributed. The hepatocytes have typical cuboidal shape, normal size, and cell membrane integrity as shown in Figure 3.

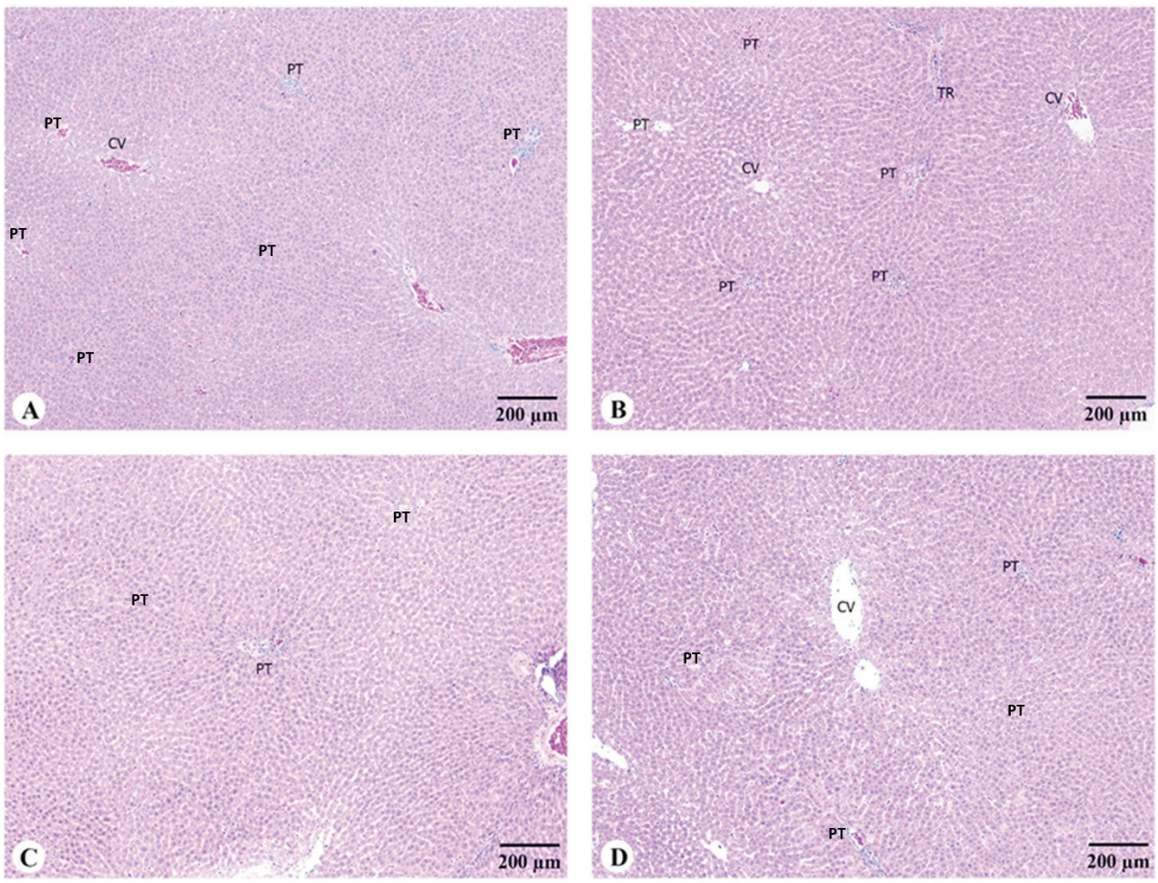

Figure 2 The Liver histological sections stained with hematoxylin and eosin of the control (A) and OA treated rats: 100 (B), 200 (C), and 400 (D) mg/kg BW. (the 4x magnification).

Note: CV; central vein, PT; portal triad, TR; trabeculae

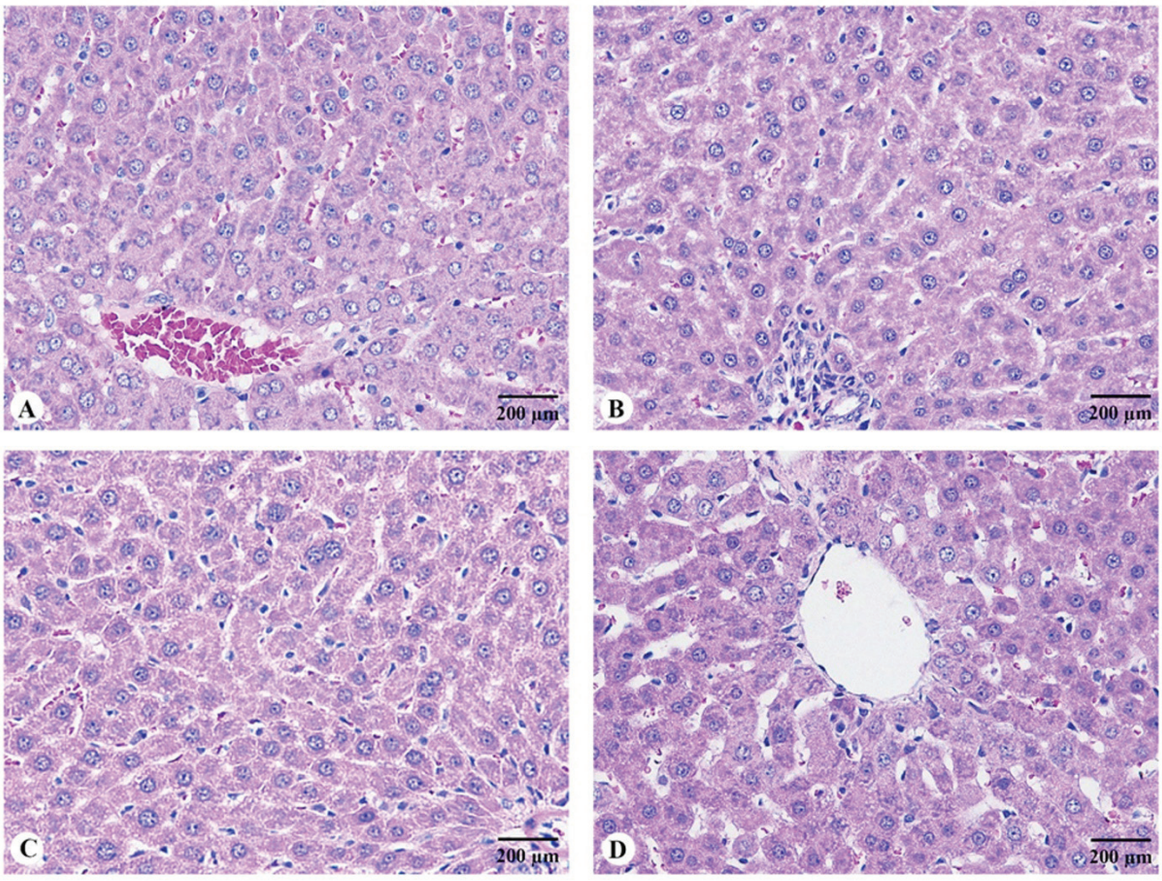

Figure 3 The Liver histological sections stained with hematoxylin and eosin of the control (A) and OA treated rats: 100 (B), 200 (C), and 400 (D) mg/kg BW (the 20x magnification).

Discussion

The presence of alkaloid, cardiac glycosides, tannin, steroid and phenolic compounds of OA leaf extract corresponds with the previous studies from Indian OA.[16] Alkaloid, tannin, and phenolic compounds possess a wide range of biological properties including antioxidant properties.[21, 22] The radical-scavenging activity of the phenolic compound is attributed to a replacement of hydroxyl groups in the aromatic ring systems of the phenolic compounds as a result of their hydrogen donating ability.[22] However, our OA extract does not contain flavonoid and saponin which were reported in previous studies. Therefore, comparative studies involving phytochemical contents from different regions should be done to determine the variation of bioactive composition among different regions.

In DPPH assay, OA extract markedly abates DPPH, although it is less effective than BHT, a commercial antioxidative substance. This result reveals that OA extract has free radical scavenging activity from the ability to give hydrogen to free radical DPPH•. The results are well correlated with an intense protein band of antioxidant enzymes, γ-GCL in the liver. γ-GCL is a cytosolic enzyme required for γ-glutamylcysteine synthesis which competently disposes of H_2O_2 by acting as glutathione peroxidase-1 cofactor.[11] Therefore OA extract, with a strong γ-GCL protein band, could play a part in cellular redox control in antioxidant activity.

Moreover, treatment with OA extract is not toxic, which is revealed by normal serum biochemical parameters along with normal histology of liver tissue. In conclusion OA extract, possesses antioxidant effects due to the presence of bioactive compounds such as alkaloid, tannin and phenolic compounds, as well as an ability to promote γ-GCL function in GSH synthesis with no harmful effects.

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Compliance with Ethics Requirements: Ethical committee clearance was obtained from Institutional care and use committee of Thammasat University (IACUC No. 009/2558). Experiments were conducted under Animals for Scientific Purposes Act, B.E. 2558 (A.D. 2015), Government of Thailand. All institutional and national rules for the care and utilization of laboratory animals were followed.

Conflict of interest: All authors report no conflicts of interest relevant to this article.

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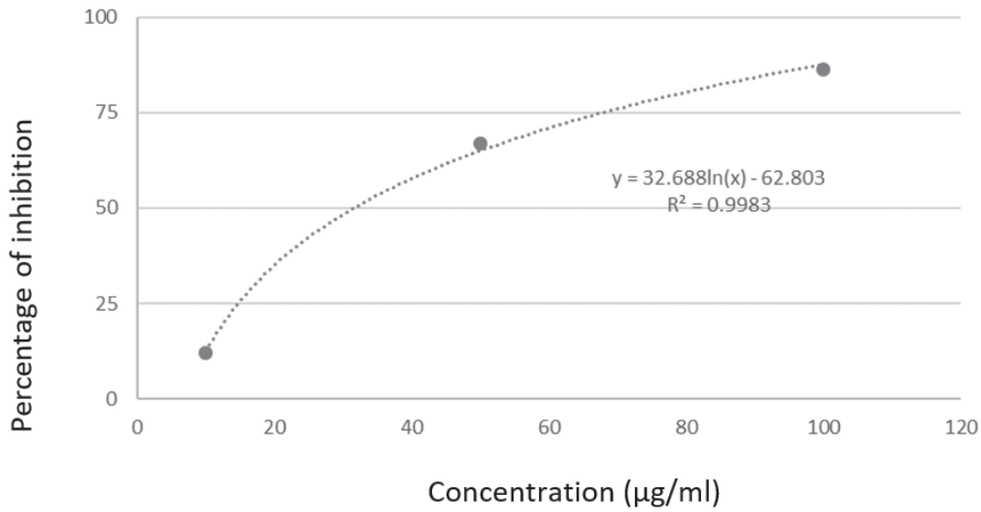
Supplementary data



Treatment	Concentration (µg/ml)	Inhibition (%)	IC_{50} (µg/ml)
OA	10	11.93 ± 0.30	36.91 ± 0.66
	50	66.84 ± 3.29	
	100	86.49 ± 0.80	
BHT			12.34 ± 1.14

Graph of DPPH radical scavenging activity of OA as the sample and BHT as the positive control. The data of percentage inhibition were expressed as mean ± SD with triplicate (n = 3)

Original Article

Efficacy and Safety of Medical Treatment in Acute Angle Closure Glaucoma at Thammasat Hospital

Sutee Ananprasert, Chayanee Penpian*

Abstract

Introduction: Acute angle closure glaucoma (AACG) is a suddenly high intraocular pressure (IOP) from pupillary blocking which may cause optic neuropathy. The key concept of AACG management is to break the pupillary block and lower the IOP in order to prevent blindness. The IOP-lowering medical treatments are prescribed to reduce the IOP in the initial stage. They are used to help clear up the cornea and reduce ocular inflammation before performing laser-peripheral iridotomy (L-PI).

Objectives: Determine the efficacy and safety of medical treatments in the initial management of AACG at Thammasat University Hospital.

Methods: Prospective descriptive study. Twelve cases of AACG were diagnosed at Thammasat Hospital. All participants were enrolled in this study. Patients without a history of drug allergy underwent a protocol of management in which they received one tablet of oral acetazolamide (250 mg), followed by one tablet every six hours, pure oral glycerin one gram per kilogram of body weight taken one time, topical timolol (0.5%) twice daily and brimonidine (0.2%) twice daily which was applied to the affected eye. The IOP were recorded at regular intervals. Medication was provided until resolution of AACG, which is defined as IOP <= 30 mmHg and resolution of acute symptoms. Qualitative data were calculated as percentages. Quantitative data were calculated as mean and standard deviation.

Results: Fifteen eyes of 12 patients, 5 (41.67%) men with the mean age of 67.58 (± 6.4) years were studied. With medical therapy, AACG resolved within 1, 12, and 24 hours are 7 (46.67%), 5 (33.33%), and 2 (13.33%), respectively. No serious adverse effects of IOP-lowering medical treatment were observed. Successful L-PI was performed in all subjects.

Conclusions: Prescription of IOP-lowering medical treatment is efficient and safe for the initial stage of IOP reduction. Medical management of AACG should still remain as the first-line treatment.

Keywords: Acute angle closure glaucoma, Medication treatment, Medication

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Department of Ophthalmology, Thammasat University Hospital, Thailand
***Corresponding author:** Chayanee Penpian, Department of Ophthalmology, Thammasat University Hospital, Thailand,
Email: chayanee.oph@yahoo.com

Introduction

Acute angle closure glaucoma (AACG) is usually caused by a relatively sudden blockage of the trabecular meshwork by the iris in the anterior chamber angle that leads to a sudden rise in intraocular pressure (IOP).[1,2,4] It is understood that the blockage of anterior chamber angle disturbs the natural aqueous flow from the posterior to the anterior chamber, creating a pressure gradient[6] and leads to a forward moving of the peripheral iris.[3,5] AACG is typically manifested by blurred vision, ocular pain, headache, and seeing halos around lights.[7] Acute systemic symptoms may result in nausea and vomiting. Signs of AACG include high IOP, mid-dilated, sluggish, and irregularly shaped pupil, corneal epithelial edema, congested episcleral and conjunctival blood vessels,[12] and shallow peripheral anterior chamber. The severe and sudden IOP elevation can irreversibly damage the optic nerve,[8] resulting in AACG.[9] The key concept of AACG management is to promptly lower the IOP in order to prevent patient from blindness.[8,10]

AACG treatment involves the admini stration of topical and systemic medications,[10] laser peripheral iridotomy (L-PI) or surgical iridectomy, and cataract surgery or glaucoma surgery.[9] The medical treatments are prescribed to reduce the IOP in the initial stage, which are used to help clear up the cornea and reduce ocular inflammation before preforming L-PI.[10] L-PI is not only the treatment of choice for pupillary-block angle closure but also prevents future AACG.[9,11]

Medical therapy of AACG is itself associated with some ocular and systemic adverse effects. The purpose of this study is to determine the efficacy and safety of medical treatment in the initial management of AACG at Thammasat University Hospital.

Methods

This prospective descriptive study followed the tenets of Helsinki declaration and was approved by the Human Research Ethics Committee of Faculty of Medicine, Thammasat University, Thailand. All participants enrolled in the study were patients diagnosed with AACG at Thammasat Hospital between July 2021 and June 2022. All of the patients were verbally informed about the treatment processes and a written inform consent was obtained from each of them.

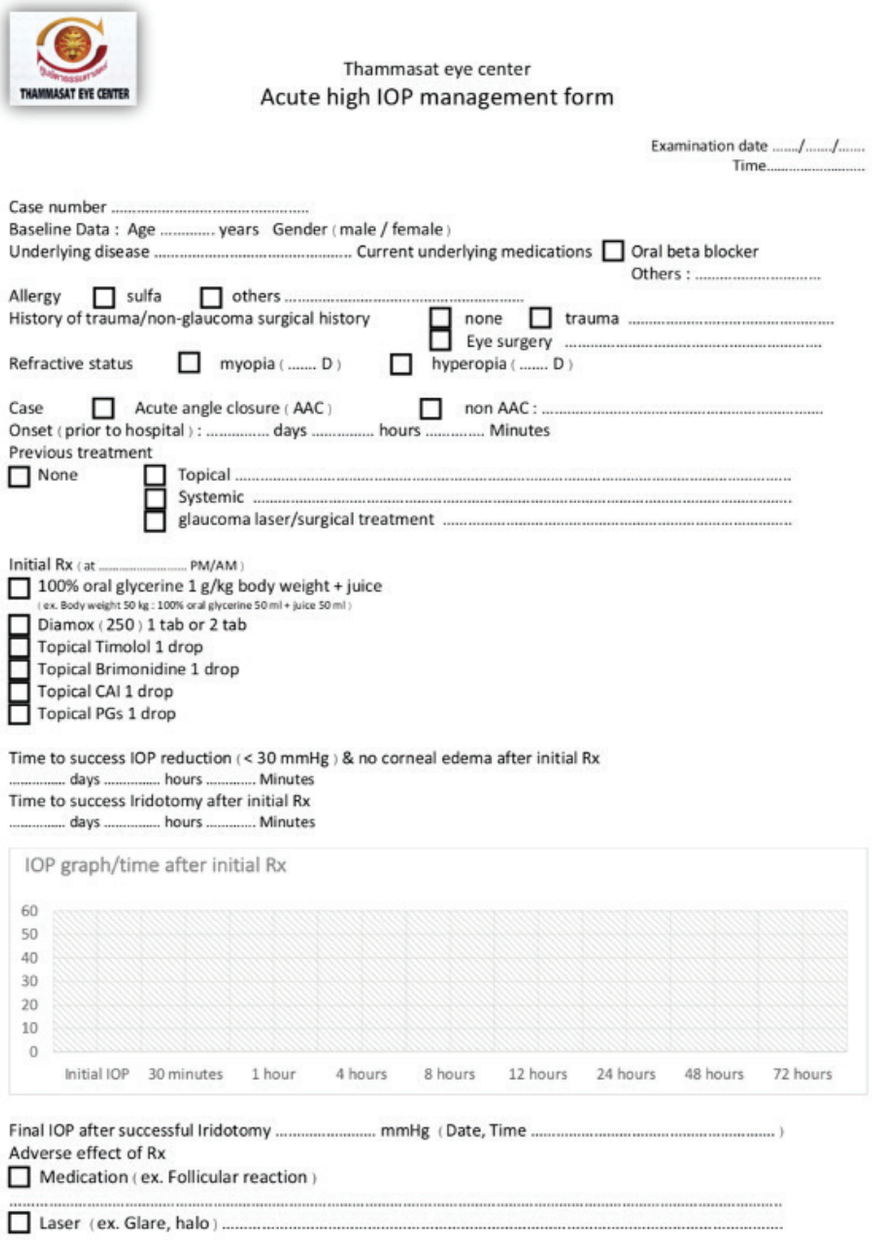
The following criteria were used to define cases of AACG:

1. Presence of at least one of the following symptoms: blurred vision, ocular pain, headache, nausea and/or vomiting, and/or seeing halos around lights.

2. Presence of IOP > 30 mmHg (as measured by Goldmann applanation tonometry) in phakic eyes with the presence of at least one of the following signs: mid-dilated, sluggish, and irregularly shaped pupil, corneal epithelial edema, congested episcleral and conjunctival blood vessels, and shallow peripheral anterior chamber.

On diagnosis, patients without a history of drug allergy underwent a protocol of management in which they received one tablet of oral acetazolamide (250 mg), followed by one tablet every six hours daily, pure oral glycerin one gram per kilogram of body weight taken one time, topical timolol (0.5%) twice daily and brimonidine (0.2%) twice daily which is applied on the affected eye. Patients were monitored at regular intervals for IOP control (as shown in Figure 1), and patients were given medication until resolution of AACG, defined as IOP <= 30 mmHg and resolution of acute symptoms. All subjects were observed for complications and adverse effects of the medical treatment. Exclusion criteria were participants with the presence of other conditions that increase IOP: for example uveitis, intraocular tumor, and patients who have history of prior intraocular surgery other than glaucoma surgery.

Age, gender and baseline data of patients were recorded in the acute high IOP management form, the IOP was checked at a specific time before and after medical treatments were prescribed until AACG was resolved. Method of treatment, complication of the treatment and adverse effect were collected (Figure 1). Qualitative data are presented as percentage. Quantitative data are calculated as mean and standard deviation.



Thammasat eye center

Acute high IOP management form

Examination date ...../...../.....
Time.....

Case number .....
Baseline Data : Age ..... years Gender (male / female)
Underlying disease ..... Current underlying medications ☐ Oral beta blocker
Others : .....
Allergy ☐ sulfa ☐ others .....
History of trauma/non-glaucoma surgical history ☐ none ☐ trauma .....
☐ Eye surgery .....
Refractive status ☐ myopia (..... D) ☐ hyperopia (..... D)

Case ☐ Acute angle closure (AAC) ☐ non AAC : .....
Onset (prior to hospital) : ..... days ..... hours ..... Minutes
Previous treatment
☐ None ☐ Topical .....
☐ Systemic .....
☐ glaucoma laser/surgical treatment .....

Initial Rx (at ..... PM/AM)
☐ 100% oral glycerine 1 g/kg body weight + juice
(ex. Body weight 50 kg : 100% oral glycerine 50 ml + juice 50 ml)
☐ Diamox (250) 1 tab or 2 tab
☐ Topical Timolol 1 drop
☐ Topical Brimonidine 1 drop
☐ Topical CAI 1 drop
☐ Topical PGs 1 drop

Time to success IOP reduction (< 30 mmHg) & no corneal edema after initial Rx
..... days ..... hours ..... Minutes
Time to success Iridotomy after initial Rx
..... days ..... hours ..... Minutes

IOP graph/time after initial Rx

60
50
40
30
20
10
0

Initial IOP 30 minutes 1 hour 4 hours 8 hours 12 hours 24 hours 48 hours 72 hours

Final IOP after successful Iridotomy ..... mmHg (Date, Time .....)
Adverse effect of Rx
☐ Medication (ex. Follicular reaction)
.....
☐ Laser (ex. Glare, halo) .....

Figure 1 AACG record form

Results

Twelve cases (15 eyes) of AACG were diagnosed and enrolled in this study. There was a predominance of women (7 [58.33%]) with the mean age of 67.58 ± 6.4 years (as shown in Table 1). The mean of presenting IOP was 45.67 ± 11.6 mmHg and mean duration of symptoms was 1.4 ± 3.7 days.

Table 1 Patient Demographic Characteristics of AACG subjects

Data	
N (%)	12(100)
Sex, NO.(%)	
Male	5(41.67)
Female	7(58.33)
Age (years old)	
40-49	0(0)
50-59	0(0)
60-69	8(66.67)
70-80	4(33.33)
Mean age	67.58 ± 6.4
Max, Min	79,62
Mean presenting IOP (mmHg)	45.67 ± 11.6
Duration of symptoms (days)	1.4 ± 3.7

With medical therapy, the number of AACG resolved within 1, 12, and 24 hours were 7 (46.67%), 5 (33.33%), and 2 (13.33%), respectively. There were no serious adverse effects of IOP-lowering medical treatment in this study. Successful L-PI was performed in all subjects within 1 to 3.5 days after the medical treatment.

There was a failure of resolution of AACG within 24 hours for only one subject (6.67%), who had symptoms persisted for more than three days before arrival. The presenting IOP in this patient was 50 mmHg. This patient finally required L-PI within 84 hours of admission. There were factors affecting time taken for resolution of the AACG within or more than 24 hours (Table 2).

Table 2 Factors affecting resolution time of AACG (within or more than 24 hours)

Factors	**Within 24 hours (%)**	**More than 24 hours (%)**
Gender		
Male	4(33.33)	1(8.33)
Female	7(58.33)	0(0)
Age		
60-69	7(58.33)	1(8.33)
70-80	4(33.33)	0(0)
Presenting IOP (mmHg)		
30-60	9(60)	0(0)
>60	5(33.33)	1(6.67)
Symptoms duration		
Within 3 days	14(93.33)	0(0)
More than 3 days	0(0)	1(6.67)

Discussion

From this study, conservative management with anti-glaucoma medication is effective and safe for relieving initial high IOP. Moreover, the majority of our patients (93.33%) resolved from AACG within 24 hours without serious adverse events. There was one patient who developed nausea and vomiting after oral acetazolamide administration for half an hour, but the IOP reduction was successful, and the nausea and vomiting disappeared after stopping oral acetazolamide.

There was one subject that the IOP-lowering medications did not work due to the patient's delayed visit to the hospital. The late arrival resulted in the prolonged IOP elevation which may stimulate inflammatory cytokine production in the anterior chamber. Inflammation may in turn influence aqueous humor dynamics and thus IOP elevation. Therefore, this patient needs to be managed by L-PI in order to lower the IOP.

Important key factors for the treatment protocol of AACG at Thammasat Hospital are hospitalization and absolute bed rest. Placing the patient in the supine position for at least a day showed additional positive results in lowering IOP since lying in supine position allows vitreous and lens-iris diaphragm to move posteriorly after antiglaucoma medication was applied in AACG patients. Furthermore, patients also had good compliance taking medications from the medical provider thus allowing a better resolution for AACG patients.

A limitation of this study is small sample size and the population studied was of all Thai descent. Moreover, as a consequence of the COVID-19 pandemic, the number of participants joining the pre-experimental study was limited due to the limit of cases as part of the hospital's protocol. Nonetheless, the information may be beneficial for patients. Initial IOP-lowering medical treatment in AACG has great benefits without serious adverse effects, and should be considered as a first-line treatment.

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Original Article

In Vitro Anti-oxidation, Anti-inflammatory and Anti-aging Activities of Oak Extract

Sayuri Kitagawa[1], Jack Jing Lin Wong[2], Tatsuo Matsuoka[1*], Yoshihiro Nakao[1], Masaaki Nakai[1]

Abstract

Introduction: Intrinsic and extrinsic factors can influence skin aging. Intrinsic skin aging can occur due to the formation and accumulation of reactive oxygen species, furthermore UV radiation contributes up to 80% of extrinsic aging. Anti-oxidation bioactive components from natural sources have been recommended for skin aging prevention.

Objectives: We explored the anti-oxidation, anti-inflammatory and anti-aging activities of oak extract (freeze-dried) product.

Methods: Anti-oxidation activity was determined using superoxide dismutase (SOD) activity assay and radical scavenging activity assay (DPPH). Anti-inflammatory activity was determined by hyaluronidase activity inhibitory assay and hexosaminidase release inhibitory assay. Anti-aging activity was determined using inhibitory assay of human neutrophil elastase activity, inhibitory assay of matrix metalloproteinase-1 (MMP-1) and promoting assay of hyaluronic acid production.

Results: Anti-oxidation activity was observed for oak extract in both SOD activity assay and radical scavenging activity assay with IC_{50} of 2.27µg/mL and 3.25 µg/mL respectively. Anti-inflammatory activity was detected in hyaluronidase activity inhibitory assay with an IC_{50} of >400µg/ml and hexosaminidase release inhibitory effect was observed with IC_{50} of 223.4 µg/mL. The oak extract also exhibited anti-aging activity with human neutrophil elastase activity inhibitory at an IC_{50} of 20.87µg/mL and MMP-1 activity inhibitory effect at an IC_{50} of 125.7µg/mL. Oak extract did not promote any hyaluronic acid production in epidermal keratinocytes.

Conclusions: Oak extract exhibits a strong antioxidant effect comparable to control Baicalin and ascorbic acid. Together with the inhibitory effect on human neutrophil elastase and MMP-1, these results suggest that oak extract can be an ingredient or part of a formula of a product to be used as a topical anti-aging agent.

Keywords: Anti-aging, Oak extract, Anti-oxidation, Anti-inflammatory

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[1] SUNTORY WELLNESS LIMITED, Japan
[2] SUNTORY WELLNESS Asia Pacific Pte. Ltd, Singapore
***Corresponding author:** Tatsuo Matsuoka, SUNTORY WELLNESS LIMITED , Japan. Email: Tatsuo_Matsuoka@suntory.co.jp

Introduction

Facial skin aging is a prevalent cosmetic concern that troubles all genders. Skin aging may manifest as wrinkles, skin sagging, dark spots, uneven skin tone and these could significantly affect an individual's self-esteem, confidence, and interpersonal relationships[1]. Both intrinsic and extrinsic factors can influence skin aging. Intrinsic factors can occur due to formation and accumulation of reactive oxygen species (ROS). Extrinsic factors such as UV radiation, alcohol usage, smoking, diet and pollution could exacerbate and accelerate skin aging. Furthermore, UV radiation can contribute up to 80% of extrinsic skin aging[2,3].

Judged to have fewer side effects, treatment with topical anti-oxidation natural bioactive components offers a noninvasive approach for skin aging prevention.

Since ancient times, the oak tree has intermingled with several fundamental aspects of human life. From providing shelter, transportation across the ocean, to the aging of fine whiskey, humans have uncovered multiple protective benefits of the esteemed oak tree. Oak trees and their parts or extracts have also been used extensively in folk medicine. The acorn has been used to treat colitis, stomatitis and diarrhea[4,5]. The leaves, bark and wood have also been investigated for their anti-microbial[6], anti-metastatic[7], anti-depressant, and anti-anxiolytic potential[8]. In this study, we set out to explore the anti-oxidation, anti-inflammatory and anti-aging activities of oak extract (freeze-dried) product to extend the protective function of oak extract as an anti-aging skin care ingredient.

Methods

- Oak extract (freeze-dried) product

Oak extract, extracted with 50% ethanol from Quercus robur Linne (Fagaceae) trees, was purchased from Maruzen Pharmaceuticals (Hiroshima, Japan) and used for all experiments in this study. Test samples were dissolved in DMSO solution and diluted in medium or buffer to test concentrations.

- Antioxidant (Suppression of Reactive Oxygen Species)

• Superoxide dismutase (SOD) Activity Assay[9]

2.4 mL of 0.05 mol/L sodium carbonate buffer (pH 10.2), 0.1 mL of 3 mmol/L xanthine, 0.1 mL of 3 mmol/L EDTA, 0.1 mL of 1.5 mg/mL bovine serum albumin and 0.1 mL of 0.75 mmol/L nitroblue tetrazolium were added to test tube, where 0.1 mL of test sample solution was added and left for 10 minutes at 25 °C. 0.1 mL of xanthine oxidase solution was added and stirred quickly, and the mixture was allowed to react at 25 °C for 20 minutes. Thereafter, 0.1 mL of 6 mmol/L copper chloride was added to stop the reaction. The absorbance at a wavelength of 560 nm was deter mined. A blank test was performed in the same manner for correction.

The method for calculating the superoxide elimination rate is as follows.

$$\text{Elimination rate (\%)} = \{1 - (A - B) / (C - D)\} \times 100$$

A: Absorbance of the test sample solution at a wavelength of 560 nm

B: Absorbance of the test sample solution (blank) at a wavelength of 560 nm

C: Absorbance at a wavelength of 560 nm in the control solution

D: Absorbance at a wavelength of 560 nm of the control solution (blank)

• Radical-Scavenging Activity Assay (DPPH)[10]

3 mL of test sample solution was added to 3 mL of 150 μmol/L diphenyl-*p*-picrylhydrazyl (DPPH) ethanol solution and incubate for 30 minutes. After that, absorbance at a wavelength of 520 nm was determined. A blank test was performed in the same manner for correction.

The calculation method of DPPH radical elimination rate is as follows.

$$\text{DPPH Elimination Rate (\%)} = \{A - (B - C)\} / A \times 100\}$$

A: Absorbance at a wavelength of 520 nm in the control solution

B: Absorbance of the test sample solution at a wavelength of 520 nm

C: Absorbance of the test sample solution (blank) at a wavelength of 520 nm

- Anti-inflammatory

• Hyaluronidase activity inhibitory assay[11]

0.1 mL of hyaluronidase solution (Type IV-S) (from bovine testis; SIGMA 400 NF units/mL))

and 0.2 mL of 0.1 mol/L acetic acid buffer (pH 3.5) was added to the test sample and incubated at 37 °C for 20 minutes. After that, 0.2 mL of 2.5 mmol/L calcium chloride was added as an activator and incubated at 37°C for 20 minutes. Then, 0.5 mL of 0.8 mg/mL sodium hyaluronate solution (from rooster comb) was added to the solution and incubated again at 37 °C for 40 minutes. Thereafter, 0.2 mL of 0.4 mol/L sodium hydroxide was added to stop the reaction, and then 0.2 mL of boric acid solution was added to each reaction solution and boiled for 3 minutes. After ice cooling, 6 mL of p-DABA reagent was added and the samples were incubated at 37 °C for 20 minutes. Thereafter, the absorbance at a wavelength of 585 nm was determined. A blank test was performed in the same manner for correction.

The calculation method of hyaluronidase activity inhibition rate is as follows.

Hyaluronidase activity inhibition rate (%)
= {1 - (A - B) / (C - D)} × 100

A: Absorbance of the test sample solution at a wavelength of 585 nm

B: Absorbance of the test sample solution (blank) at a wavelength of 585 nm

C: Absorbance at a wavelength of 585 nm in the control solution

D: Absorbance at wavelength 585 nm of control solution (blank)

- **Hexosaminidase Release Inhibitory Assay**[12]

Rat basophil leukemia cells (RBL-2H3) were cultured using 15% FBS-added S-MEM and collected by trypsinization. Recovered cells were diluted in medium to a concentration of 4.0×10^5 cells/mL and DNP-specific IgE was added such that the mixture will have a final concentration of 0.5 μg/mL of DNP-specific IgE. 100 μL mixture was plated onto each well of the 96-well plate and cultured overnight. The medium was next removed, and the cell was washed twice with 100 μL of Siraganian buffer. Next, 30 μL of the buffer and 10 μL of the test sample prepared in the Siraganian buffer were added and allowed to stand at 37 °C for 10 minutes. Thereafter, 10 μL of 400 ng/mL DNP-BSA solution was added and allowed incubate at 37 °C for 15 minutes to release hexosaminidase. Later, the reaction was stopped by placing the 96-well plate on ice. 10 μL of cell supernatant of each well and 10 μL of 1 mmol/L p-nitrophenyl N-acetyl-D-glucosaminide β (p-NAG) solution was added to a new 96-well plate and incubated at 37 °C for 1 hour. After completion of the reaction, 250 μL of 0.1 mol/L Na_2CO_3 / $NaHCO_3$ were added to each well, and the absorbance at wavelength 415 to 650 nm was determined. Blank tests include 10 μL of cell supernatant, 10 μL of citrate buffer and 250 μL of 0.1 mol/L Na_2CO_3/$NaHCO_3$ mixture, where absorbance at wavelength 415 to 650 nm was determined and corrected.

The method for calculating the hexosaminidase release inhibition rate is as follows.

Hexosaminidase release inhibition rate (%)
= {1 - (B / A)} × 100

A: Absorbance at wavelength 415 to 650 nm without test sample added

B: Absorbance at wavelength 415 to 650 nm with test sample added

- **Anti-aging**

- **Inhibitory Assay of Human Neutrophil Elastase Activity**[13]

50 μL test sample prepared in 0.1 mol/L HEPES buffer (pH 7.5) was mixed with 25 μL of 6 μg/mL elastase (Human Leukocyte) solution in a 96-well plate. Thereafter, 25 μL of 2 mmol/L N-METHOXYSUCCINYL-ALA-ALA-PRO-VAL-p-NITRO-ANILIDE solution prepared with the above buffer solution was added and incubated at 25 °C for 15 minutes. After completion of the reaction, absorbance at a wavelength of 415 nm was determined. A blank test was performed in the same manner for correction.

The method for calculating the elastase activity inhibitory rate is as follows.

Elastase activity inhibition rate (%)
= {1 -(C - D)/(A - B)} × 100

A: Absorbance at wavelength 415 nm without test sample added but enzymes added

B: Absorbance at wavelength 415 nm without test sample and enzymes added

C: Absorbance at wavelength 415 nm with test sample added and enzyme added

D: Absorbance at wavelength 415 nm with test sample added but no enzymes added

- **Inhibitory Assay of MMP-1**[14]

This test method is a partial modification of the Wunsch and Heidrich method. 50 μL of test

sample that was dissolved in 0.1 mol/L Tris-HCl buffer (pH 7.1) containing 20 mmol/L calcium chloride was mixed with MMP-1[(a)] solution 50 µL and 400 µL of Pz-peptide [(b)] solution. After incubating at 37°C for 30 minutes, the reaction was stopped with 1 mL of 25 mmol/L citric acid solution. Thereafter, 5 mL of ethyl acetate was added and vigorously shaken. This was centrifuged (1600 × g, 10 min) and the absorbance of the ethyl acetate layer at a wavelength of 320 nm was determined. A blank test was performed in the same manner for correction.

The calculation method of the MMP-1 activity inhibitory rate is as follows.

MMP-1 activity inhibition rate (%)
= {1 - (C - D) / (A - B)} × 100

A: Absorbance at wavelength of 320 nm without test sample but with enzymes added

B: Absorbance at a wavelength of 320 nm without both test sample and enzymes added

C: Absorbance at wavelength of 320 nm with both test sample and enzyme added

D: Absorbance at wavelength of 320 nm with test sample added but no enzymes added

(a) MMP-1: COLLAGENASE Type IV from Clostridium histolyticum (Sigma)

(b) Pz-peptide: Pz-Pro-Leu-Gly-Pro-D-Arg-OH (BACHEM Feinchemikalien AG)

- **Promoting Assay of Hyaluronic Acid Production (Epidermal Keratinocytes)**[15,16]

Normal human neonatal epidermal keratinocytes (NHEK) were cultured using normal human epidermal keratinocyte proliferation medium (KGM), and then cells were collected by trypsin treatment. The recovered cells were diluted with KGM to a concentration of 1×10^5 cells/mL and plated at a volume of 100 µL per well in a 96-well plate and cultured for 24 hours. After which 100 µL of test sample dissolved in KGM was added to each well and cultured for 3 days. The amount of hyaluronic acid in the medium of each well was determined by the sandwich method using hyaluronic acid-binding protein (HABP).

The calculation method of the hyaluronic acid production promotion rate is as follows.

Hyaluronic acid production promotion rate (%) = (A / B) × 100

A: Amount of hyaluronic acid with test sample added

B: Amount of hyaluronic acid without test sample added

- **MTT Assay**

Cell viability in this study was confirmed using the MTT assay. After 2 hours of incubation, the blue formazan produced in the cells was extracted with 100 µL of 2-propanol. After extraction, measured the absorbance at 570 nm and at 650 nm as turbidity, and counted the difference between the two as the amount of blue formazan produced. The difference between blue formazan production in the cells with and without the test sample was evaluated.

Statistical analysis

All statistical analyses were carried out using the paired t-test and compared with the control (no sample added). A *p-values* <0.05 were considered statistically significant. Data in the Tables are shown as mean ± SEM.

Results

- Antioxidant effects (Suppression of Reactive Oxygen Species)

- **Effect of Oak Extract on Superoxide dismutase (SOD) Activity Assay**

Oak extract exhibited a strong anti-oxidation effect in SOD activity assay. In the same experiment, compared to positive control Baicalin, Oak extract was more effective with a determined IC_{50} of 2.27 µg/mL compared to 3.94 µg/mL of the positive control. (Table 1)

Table 1 Effect of Oak Extract on SOD Activity

Concentration of tested sample (µg/mL)	Elimination rate (%) of Oak Extract Product
0	0.0 ± 3.3
1.56	**37.6 ± 0.8*****
6.25	**80.1 ± 0.2*****
25	**94.4 ± 0.1*****
IC_{50} (µg/mL)	**2.27**

Positive control Baicalin; IC_{50} = 3.94 µg/mL
Mean ± S.E., n = 3, ***: $p<0.001$

• **Effect of Oak Extract on Radical-Scavenging Activity Assay (DPPH)**

Oak extract exhibited a strong radical-scavenging activity in DPPH assay. Compared to positive control, ascorbic acid, a well-known anti-oxidant, the IC_{50} of Oak extract was determined to be 3.25 µg/mL comparable to the IC_{50} of 3.04 µg/mL ascorbic acid. (Table 2)

Table 2 Effect of Oak Extract on DPPH elimination

Concentration of Tested sample (µg/mL)	DPPH Elimination Rate (%) of Oak Extract Product
0	0.0 ± 1.2
3.125	**48.2 ± 1.1*****
6.25	**84.2 ± 0.1*****
12.5	**92.4 ± 0.0*****
IC_{50} (µg/mL)	**3.25**

Positive control ascorbic acid; IC_{50} = 3.04 µg/mL
Mean ± S.E., n = 3, ***: $p<0.001$

- **Anti-inflammatory effects**

• **Effect of Oak Extract in hyaluronidase activity inhibitory assay**

Oak extract exhibited a weak anti-inflammatory action on hyaluronidase activity inhibitory assay. Compared to indomethacin IC_{50} of 30.3 µg/mL, Oak extract has an IC_{50} of more than 400 µg/mL. (Table 3)

Table 3 Effect of Oak Extract on hyaluronidase activity inhibitory assay

Concentration of Tested sample (µg/mL)	Hyaluronidase Activity Inhibition Rate (%) of Oak Extract Product
0	0.0 ± 0.2
25	-12.6 ± 3.5
100	1.8 ± 1.6
400	**24.7 ± 0.7*****
IC_{50} (µg/mL)	**>400**

Control indomethacin; IC_{50} = 30.3 µg/mL
Mean ± S.E., n = 3, ***: $p<0.001$

• **Effect of Oak Extract on Hexosaminidase Release Inhibitory Assay**

Oak extract exhibited a weak anti-inflammatory action on hexosaminidase release inhibitory Assay. Compared to indomethacin IC_{50} of 101.8 µg/mL, Oak extract has an IC_{50} of 223.4 µg/mL. (Table 4)

Table 4 Effect of Oak Extract on Hyaluronidase activity inhibitory assay

Concentration of Tested sample (µg/mL)	Hexosaminidase Release Inhibition Rate (%) of Oak Extract Product
0	0.0 ± 6.0
100	8.5 ± 3.2
200	**43.6 ± 1.0****
400	**75.5 ± 1.3*****
IC_{50} (µg/mL)	**223.4**

Control indomethacin; IC_{50} = 101.8 µg/mL
Mean ± S.E., n = 3, **: p<0.01, ***: p<0.001

- Anti-aging effects

• **Effect of Oak Extract on Inhibitory Assay of Human Neutrophil Elastase Activity**

Anti-aging effect was observed for Oak extract on inhibitory assay of human Neutrophil Elastase activity with an IC_{50} of 20.87 µg/mL. This is effect is stronger compared to *Hypericum Ascyron* flowers an ingredient with established IC_{50} of 150 µg/mL[17]. (Table 5)

Table 5 Effect of Oak Extract on Inhibitory Assay of Human Neutrophil Elastase Activity

Concentration of Tested sample (µg/mL)	Elastase Activity Inhibition Rate (%) of Oak Extract Product
0	0.0 ± 8.2
6.25	23.7 ± 6.0
25	**57.4 ± 0.3****
100	**79.9 ± 0.1*****
IC_{50} (µg/mL)	**20.87**

Mean ± S.E., n = 3, **: p<0.01, ***: p<0.001

• **Effect of Oak Extract on Inhibitory Assay of MMP-1**

Inhibitory assay of MMP-1 was carried out to determine the IC_{50} of Oak Extract. A moderate inhibitory effect could be detected with an IC_{50} of 125.7 µg/mL. (Table 6)

Table 6 Effect of Oak Extract on Inhibitory Assay of MMP-1

Concentration of Tested sample (µg/mL)	MMP-1 Activity Inhibition Rate (%) of Oak Extract Product
0	0.0 ± 0.2
25	**8.7 ± 1.5***
100	**40.9 ± 0.7*****
400	**77.4 ± 0.2*****
IC_{50} (µg/mL)	**125.7**

Mean ± S.E., n = 3, *: p<0.05, ***: p<0.001

- Effect of Oak Extract on Promoting Assay of Hyaluronic Acid Production (Epidermal Keratinocytes)

Finally, we tried to detect if Oak extract has any effect in promoting hyaluronic acid production in epidermal keratinocyte, however we were not able to detect any activity. The lowest hyaluronic acid production rate at the highest concentration (100 μg/mL) was due to a decrease in cell viability to 10% (confirmed by MTT assay). (Table 7)

Table 7 Effect of Oak Extract on Promoting Assay of Hyaluronic Acid Production (Epidermal Keratinocytes)

Concentration of Tested sample (μg/mL)	Hyaluronic Acid Production Promotion Rate (%) of Oak Extract Product
0	100.0 ± 2.1
1.56	98.3 ± 1.2
6.25	82.1 ± 1.2
25	82.1 ± 1.9
100	67.1 ± 1.4

Discussion

Facial skin aging is a prevalent cosmetic concern that troubles all genders. Skin aging may manifest as wrinkles, skin sagging, dark spots, uneven skin tone and these could significantly affect an individual's self-esteem. UV radiation, ambient pollutant and smoking are the most prominent and prevalent external stressor affecting skin aging, the continuous exposure to these environmental stressors stimulate the production of reactive oxygen species, leading to oxidative stress[18-20]. In this study we set out to investigate if oak extract can confer any *in vitro* anti-oxidation, anti-inflammatory and anti-aging activities.

In our anti-oxidation study (Table 1 and 2) we found that oak extract was able to confer anti-oxidation effect at a level that is comparable or stronger than the control. This is consistent with other studies which had demonstrated oak genus possess antioxidant capacity[21,22] which suggests that oak extract has a great potential to be used to counter oxidative stress dealt to the skin by the environment.

Skin inflammation can manifest as redness, pain, itchiness and dryness which can be unsightly and a challenge to manage[23]. We evaluated the anti-inflammatory action in Oak extract through Hyaluronidase activity inhibitory assay (Table 3) and Hexosaminidase Release Inhibitory Assay (Table 4). Hyaluronidase is an enzyme that depolymerizes the hyaluronic acid involved in inflammation, and it has been shown that the inhibition of hyaluronidase could reduce inflammation[24]. Hexosaminidase is released by mast cells via IgE-mediated degranulation and the release mediates inflammation and acute allergy reaction[25]. While oak extract weakly inhibited hyaluronidase activity, we were able to detect hexosaminidase release inhibitory activity, therefore suggesting that oak extract product might play a role in attenuation of inflammation and acute allergy reaction.

Intrinsic skin aging can be classified as natural aging and is caused by changes in skin elasticity. Collagen, the most abundant protein in the extracellular matrix (ECM) of connective tissue in the human dermis, can be cleaved by elastase which can then cleave elastin, fibronectin and other ECM proteins, leading to a deterioration of the skin structure[26]. The degradation of elastin and collagen further induce the production of MMPs, which activates a series of destructive processes such as inflammation and aging of the skin[27]. Compared to Hypericum ascyron flowers, an ingredient with established IC_{50} of 150 μg/mL[17], oak extract product has a more potent IC_{50} (Table 5) and at the same time we were able to detect its inhibitory effect on MMP-1 (Table 6). Hyaluronic acid is another key component of the ECM and it's free-radical scavenging function makes it an attractive molecule to study for anti-aging skin care. Unfortunately, we were not able to detect any effect of oak extract on the production of hyaluronic acid in epidermal keratinocytes (Table 7), the reduction in hyaluronic acid observed was due to reduction of cell viability, which we validated with an MTT assay.

In conclusion, we have investigated and reported on the *in vitro* anti-oxidation, anti-inflam matory and anti-aging activities of oak extract through multiple experiments. Oak extract exhibits a strong antioxidant effect comparable to control Baicalin and ascorbic acid. It also exhibited an inhibitory effect on human neutrophil elastase and MMP-1. In addition to being able to confer protection on extrinsic aging factors, oak extract has the potential to inhibit intrinsic factors responsible for skin aging. These results suggest that oak extract can be an ingredient or part of a formula of a product to be used as a topical anti-aging agent.

This study has shed light on the *in vitro* activity of oak extract, in order to further establish the functionality, an *in vivo* clinical study should be considered. This study also warrants further investigation to identify the key active ingredient in oak extract.

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Original Article

Molnupiravir Metabolite--N^4-hydroxycytidine Causes Cytotoxicity and DNA Damage in Mammalian Cells *in vitro*

Pholasit Chamod[1], Sutheera Sangsiri[2], Krittin Tangjittham[3], Pattranit Liu[3], Pakapon Mongkhonsakunrit[3], Jessada Pakotiprapha[3], Tanaboon Rimdusit[3], Onmanee Prajuabjinda[4], Saovapak Poomirat[4*]

Abstract

N^4-hydroxycytidine (NHC) is the active metabolite of molnupiravir—a new drug for COVID-19 treatment. NHC exerts antiviral activity by incorporating into SAR-CoV-2 RNA leading to false base-paring and lethal mutations to the virus. However, the risk of non-specific mutagenesis to host cells has been a concern. The goal of this study is to detect cytotoxic activity and DNA damage induced by NHC in rapid-growing cells including human keratinocyte (HaCaT), and human adenocarcinomic alveolar basal epithelial (A549) cells *in vitro* by using sulforhodamine B (SRB) colorimetric and comet assays. NHC induced cytotoxicity in a concentration-dependent manner (0.1-30μM) in HaCaT and A549 cells. Half-maximal inhibitory concentration (IC_{50}) values of NHC were lower in HaCaT compared to A549 cells after 3, 5, 10 days of exposure (4.40 ± 0.09 *vs* 23.21 ± 3.42, 5.82 ± 0.91 vs 16.35 ± 2.04, and 5.41 ± 0.88 *vs* 13.83 ± 2.05 μM, respectively), suggesting that the cytotoxic effect of NHC is more potent in HaCaT cells than in A549 cells. Significant increase in DNA damage parameters were observed in comet assay for HaCaT and A549 cells after exposure to NHC. NHC-induced DNA damage in HaCaT cells was concentration-dependent (1-10 μM), and time-dependent (3-10 days). NHC-induced DNA damage in A549 cells was concentration-dependent (1-10 μM), but not time-dependent (3-10 days). Within the limitations of this *in vitro* study, we conclude that NHC could induce cytotoxic and DNA damage in mammalian cells at therapeutic and supratherapeutic concentrations. We propose caution in the use and supervision of molnupiravir, especially in patients with impaired xenobiotic clearance.

Keywords: N^4-hydroxycytidine, Molnupiravir, Cytotoxicity, DNA amage, Comet assay

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[1] Biochemistry Division, Department of Preclinical Sciences, Faculty of Medicine, Thammasat University, Pathum Thani, Thailand
[2] Pharmacology Division, Department of Preclinical Sciences, Faculty of Medicine, Thammasat University, Pathum Thani, Thailand
[3] Faculty of Medicine, Thammasat University, Pathum Thani, Thailand
[4] Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathum Thani, Thailand
***Corresponding author:** Saovapak Poomirat, Ph.D, Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathum Thani, Thailand, Email: saovapak@tu.ac.th, Tel. +66 909650547.

Introduction

Molnupiravir is a new cost-effective oral antiviral agent classified as anti-ribonucleoside analog currently in phase III clinical trials.[1] Although molnupiravir has not been officially approved owing to low safety database, and mutagenesis concern, it is authorized for emergency use for COVID-19 treatment worldwide due to the pandemic crisis and high demand for antiviral drug treatment.[2,3] Molnupiravir is a prodrug that is hydrolyzed by esterase to form an active metabolite N^4-hydroxy cytidine (NHC).[4] NHC in systemic circulation is up-taken into host cells and phosphorylated to form NHC-triphosphate.[4] This compound competes with cytidine triphosphate (CTP) or uridine triphosphate (UTP) as a substrate for the viral RNA-dependent RNA polymerase leading to lethal mutations to the SARV-CoV-2.[5-7]

Molnupiravir is contraindicated in patients younger than 18 years of age and in pregnancy, as it inhibits myeloid and erythroid proliferation, and is associated with embryofetal lethality and teratogenicity in animals at high concentration.[8] A recent study from the Zhou and colleagues showed that NHC is mutagenic in Chinese hamster ovary cells *in vitro*.[9] The missense and frame-shift deletion mutations occur in cells that are exposed to NHC.[9] In contrast, Githaka group showed that the use of molnupiravir at therapeutic doses for 5 days efficiently eliminates SARS-CoV-2 without altering the number of gene missense and frameshift variants in lung cells of golden hamsters.[10] Troth and colleagues showed that molnupiravir treatment did not increase mutation in Pig-a mutagenicity assay and Big Blue transgenic rodent assay in 5-day exposure.[11] Accumulation of toxic NHC may occur in individuals with impaired xenobiotic clearance. Genotoxicity study of supratherapeutic concentration or prolonged exposure was not performed in these studies. Here, we assessed cytotoxic and DNA damage effects of NHC in HaCaT, and A549 cells *in vitro* by using SRB and comet assay. This work could in part fulfill the safety profiles of molnupiravir.

Methods

Cell culture

The cells in this experiment consisted of two cell types: adenocarcinomic human alveolar basal epithelial cells A549 (ATCC CCL-185), and human keratinocytes HaCaT (CLS 300493-SF) cells. The A459 cells were grown in RPMI 1640 medium, which was supplemented with 10% heated fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin. The HaCaT cells were grown in DMEM medium supplemented with 10% heated fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin (Gibco BRL, Grand Island, NY, USA). The cell lines were cultured at a temperature of 37ºC with 5% CO_2 and 95% humidity.

Drug preparation

N^4-hydroxycytidine (TCI, Oregon, USA) was dissolved in DMSO making stock solution at 10 mM in small aliquots and kept at -20ºC. Working solutions of NHC contains <0.3% DMSO v/v. Final solutions were diluted in culture medium on the day of experiment.

In vitro assay for cytotoxic activity

Cytotoxic activity could be measured by using SRB assay as described previously.[12-14] The plating densities of cell lines were identified based on their growth patterns, expressed as cells per well. The plating densities of cell lines A594 were 5.5×10^3, 2.5×10^3, and 5×10^2 cells and HaCaT were 10×10^3, 8×10^3 and 3×10^3 cells for 3, 5, and 10 days of NHC exposure, respectively. These densities were chosen to encourage exponential growth during the experiment and to find a linear relationship between absorbance at 492 nm and cell count in the SRB assay. Firstly, the cell cultures were cultivated as monolayers in a 75 cm^2 flask and afterwards rinsed with phosphate buffer saline (PBS) (Sigma-Aldrich® in Saint Louis, USA). After that, PBS was removed and 0.15% trypsin-EDTA (Gibco BRL, Grand Island, NY, USA) was added to obtain a suspension of single cells in the medium. The cells were incubated for 5 to 10 minutes and then washed with 5 mL of medium in a 75 cm^2 flask to stop the trypsin-EDTA activity. The count density of cell viability was determined with trypan blue reagent using a hemocytometer. The A549 and HaCaT cell suspensions were diluted in their suitable medium to achieve their plating densities, then seeded in 96-well microplates at a volume of 100 µL per well. The microplates were then placed in an incubator at 37 ºC, with 5% CO_2 and 95% humidity for 24 hours. Subsequently, NHC was added to treat cells at various

doses ranging from 0.1 to 30 μM. The exposure times were 3, 5 and 10 days. The concentration of each NHC was added at a volume of 100 μL per well in 96-well microplates. The control medium was added to 100 μL of cell culture medium, while the control solvent was mixed with a 100 μL solution of 2% DMSO. The cell culture plates were incubated at 37°C and 5% CO_2 for 72 hours. In the case of 5 or 10 days of exposure treatment, the medium was removed after 72 hours of incubation, and the new NHC was added at various doses to each well and incubated, a process that was repeated every 72 hours until fixation time. For the fixation time, 100 μL of cold 40% trichloroacetic acid (TCA) was added directly to the medium supernatant in each well and incubate at 4ºC for 1 hour. Then, the culture plates were washed four times with slow-running tap water and gently tapped onto a paper towel to remove excess water. The plate was allowed to air dry at room temperature. 50 μL of SRB solution (0.4% w/v in 1% glacial acetic acid) was added to each well for 30 min, then washed four times with 1% acetic acid and excess water removed. The plate was allowed to dry completely at room temperature. 100 μL of 10 mM Tris base (Tris (hydroxymethyl) aminomethane, pH 10.5) was added to each well to dissolve the dry product. The absorbance (OD) was read on a microplate reader at 492 nm. The survival percentage and IC_{50} values were analyzed.[13,15]

Comet assay for detection of DNA damage

DNA strand breaks can be measured by a comet assay, a relatively simple, and sensitive method, as described previously.[16-18] The assay is based on measurements of fragmented DNA migrating out of the nucleus during electrophoresis.[17,19]

Cell treatment and isolation

The plating densities in 24-well culture plates of cell lines A594 were $1.65x10^4$, $7.5x10^3$, and $1.5x10^3$, and HaCaT were $3x10^4$, $2.4x10^4$ and $9x10^3$ for 3, 5, and 10 days of NHC exposure, respectively. A594 and HaCaT cells were treated with NHC (1 or 10 μM) at exposure time of 3, 5 and 10 days. In the case of 5 or 10 days of exposure, the medium and NHC were replaced every 72 hours of incubation until the cells were harvested by using 0.05% trypsin-EDTA (Sigma). Untreated cells were used to confirm that background damage was low. Negative controls included untreated cells cultured in normal media (control media, CM), and in normal media with DMSO (control solvent, CS with 0.3% DMSO v/v). Positive control was performed by exposing untreated cells at day-5 cultured with 20 mM hydrogen peroxide.[20,21] Cell density was adjusted to about 2×10^4 cells/mL in phosphate-buffered saline lacking divalent cations by using hemocytometer. Cell suspension of 0.4 mL were transferred into a 5-mL tube for combining with low-melting-point agarose in the next step.

Slide preparation and gel electrophoresis

Comet assay was performed under alkaline conditions according to Singh and coworkers with a few modifications.[22] Briefly, clear-glass microscopic slides (25.4×76.2×1.0 mm) were precoated with 1.0% (w/v) normal melting point (NMP) agarose (Thermo Scientific, Massachusetts, USA.), then the treated cells (~10,000 cells) were mixed with 0.5% (w/v) low melting point (LMP) agarose (Thermo Scientific, Massachusetts, USA.) and pipetted over the first layer. After drying, the third layer of LMP was pipetted over the second layer. The glass slide (24×60 mm) was then applied to cover a layered-agarose gel and allowed to solidify. Then the slides were immersed in a cold lysing solution for 2 hours at 4ºC. After lysis, DNA was allowed to unwind for 20 minutes in alkaline electrophoresis solution pH>13. Electrophoresis was performed at 4°C, 24 V/cm, and 300 mA current. The slides were then neutralized with cold 0.4 M Tris, pH 7.5, stained with ethidium bromide. Slides were scored using the Comet Imager program (Metasystems, Germany) attached to a fluorescent microscope (Olympus BX50). Comets were scored at 100x magnification. Images from at least 100 cells (50 cells from each replicate slide) were analyzed. The parameter taken to assess cell line DNA damage was tail length (μm) and % DNA in tail.

Data analysis

Data were analyzed by using Prism9 (GraphPad Software, San Diego). The data presented as mean ± SD, and the significance difference is indicated when the P-value is less than 0.05. Group data were analyzed by unpaired student t-test or ANOVA followed by multiple comparisons against control.

Results

Effects of N4-hydroxycytidine (NHC) on cell viability

NHC decreased cell viability in a concentration (0.1-30 μM)-dependent manner to HaCaT and A549 cells ($p = 0.0001$ and $p<0.0001$, respectively). Furthermore, the cytotoxicity effects remained consistent across exposure duration of 3, 5, and 10 days of exposure in both HaCaT ($p = 0.0794$, Fig.1A) and A549 cells ($p = 0.3852$, Fig.1B).

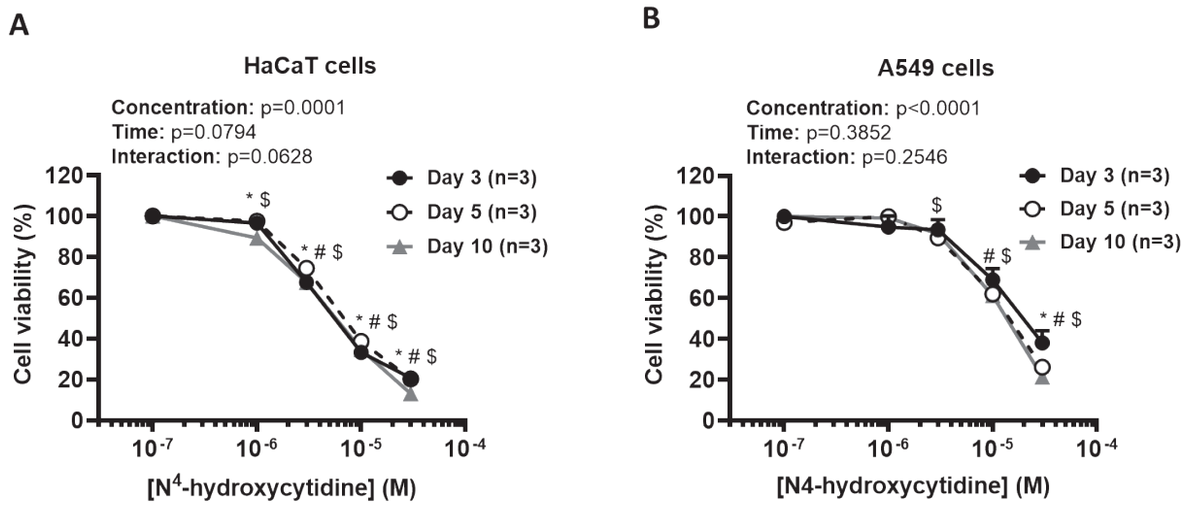

Figure 1 Cytotoxicity in response to N^4-hydroxycytidine (NHC) exposure in HaCaT (A) and A549 cells (B). Data are presented as mean ± SD; n indicated number of replicates; *, $^{\#}$, $^{\$}$ significant differences compared to the cytotoxic effect of 0.1μM NHC at 3, 5, and 10 days, respectively, as analyzed by two-way ANOVA with Dunnett's multiple comparison test.

Differential cytotoxic response to NHC of HaCaT and A549

Concentration-response curves (CRCs) of NHC (0.1-30 μM) were generated, and IC_{50} values were analyzed to compare NHC-induced cytotoxicity in HaCAT and A549 cells. The CRCs of NHC in HaCaT showed a leftward shift compared to A549 at 3, 5, and 10 days of exposure (Fig. 2A-C). The IC_{50} values of NHC in HaCaT were lower than those in A549 cells at 3, 5, 10 days of exposure (Fig. 2D). Furthermore, the IC_{50} values of A549, but not HaCaT exhibited a time-dependent decrease from 3 to 10 days (Fig. 2D). The IC_{50} values of NHC at various exposure durations in HaCaT and A549 were summarized in Table 1.

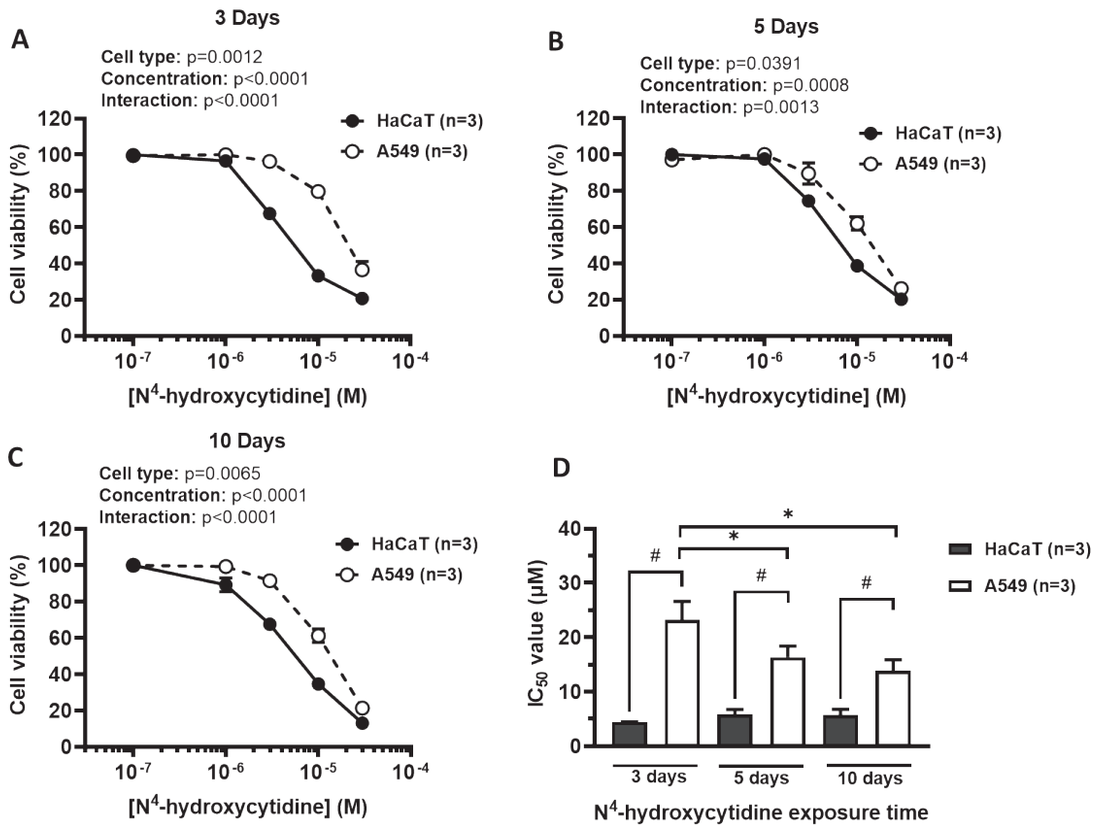

Figure 2 Differential cytotoxicity of HaCaT and A549 cells in response to NHC exposure over three different durations; 3 days (A), 5 days (B) and 10 days (C). IC_{50} of NHC against both cells after 3, 5 and 10 days of exposure (D). *significant differences among the different exposure durations, analyzed by one-way ANOVA with Tukey's multiple comparisons test. # significant differences between tested cells at each exposure duration, analyzed by unpaired t test. Data are presented as mean ± SD, n indicated number of replicates, p values shown in A-C were analyzed by two-way ANOVA.

Table 1 IC_{50} values of NHC on HaCaT and A549 cells

Cell lines	Exposure time to NHC (days)	N	IC_{50} value (µM) (Mean ± SD)	*P*-value
HaCaT (Human keratinocyte cells)				
	3	3	4.40 ± 0.09	0.128
	5	3	5.82 ± 0.91	
	10	3	5.41 ± 0.88	
A549 (Human adenocarcinoma alveolar basal epithelial cells)				
	3	3	23.21 ± 3.42	0.0107*
	5	3	16.35 ± 2.04**	
	10	3	13.83 ± 2.05**	

* significant difference among different exposure durations within the same cell type (one-way ANOVA)
** significant difference compared to day 3 exposure (one-way ANOVA with Turkey's multiple comparison test)
Data were mean ± SD; N = number of replicates.

Effect of NHC exposure on DNA damage

To assess the genotoxic activity of NHC, comet assay was performed in HaCaT and A549 cells. Comet assay is an economical and simple method using single-cell electrophoresis to detect DNA breaks in eukaryotic cells.[23] Figure 3 shows representative results of comet characteristics due to DNA damage in untreated cells (Fig. 3A) vs positive control H_2O_2 treatment (Fig. 3B). The analysis of DNA damage parameters included quantification of DNA tail length and %DNA in tail (Fig. 3C-D). Changes in DNA damage parameters after NHC exposure were quantified as shown in Fig. 4. DMSO 0.3% v/v (control solvent, CS) did not alter DNA tail length and % DNA in tail compared to control medium (CM) indicating changes in DNA damage parameters were induced by NHC. There were consistent increases in tail length and % DNA in tail of HaCaT cells exposed to NHC compared to CM in time-dependent (3-10 days), and concentration-dependent (1 and 10μM) manners (Fig. 4A-B). A549 cells exposed to NHC also showed consistent increases in DNA tail length and % DNA in tail in concentration-dependent (1 and 10 μM) manner, but not in time-dependent (3 and 10 days) manner (Fig. 4C-D).

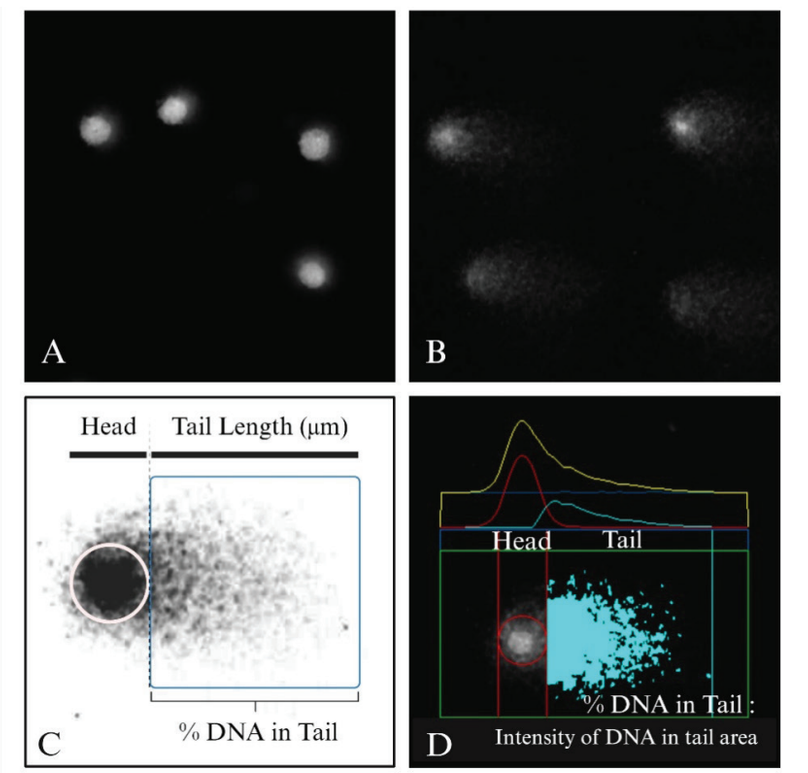

Figure 3 Representative images of alkaline comet assay in HaCaT cells. Untreated cells displayed intact round nuclei (A). Exposure to 20 mM H_2O_2 for 5 minutes caused DNA breaks, and DNA fragment migration from nucleoid body (comet-shape characteristics, (B)). Comet parameters for quantifying DNA damage were demonstrated in (C). Original color was adjusted to white background for better representation (C). Comet imager software captured fluorescence image, and individually identified cell head area, tail length and intensity of tail area (D). Tail length and % DNA in tail, were used to quantify DNA damage.

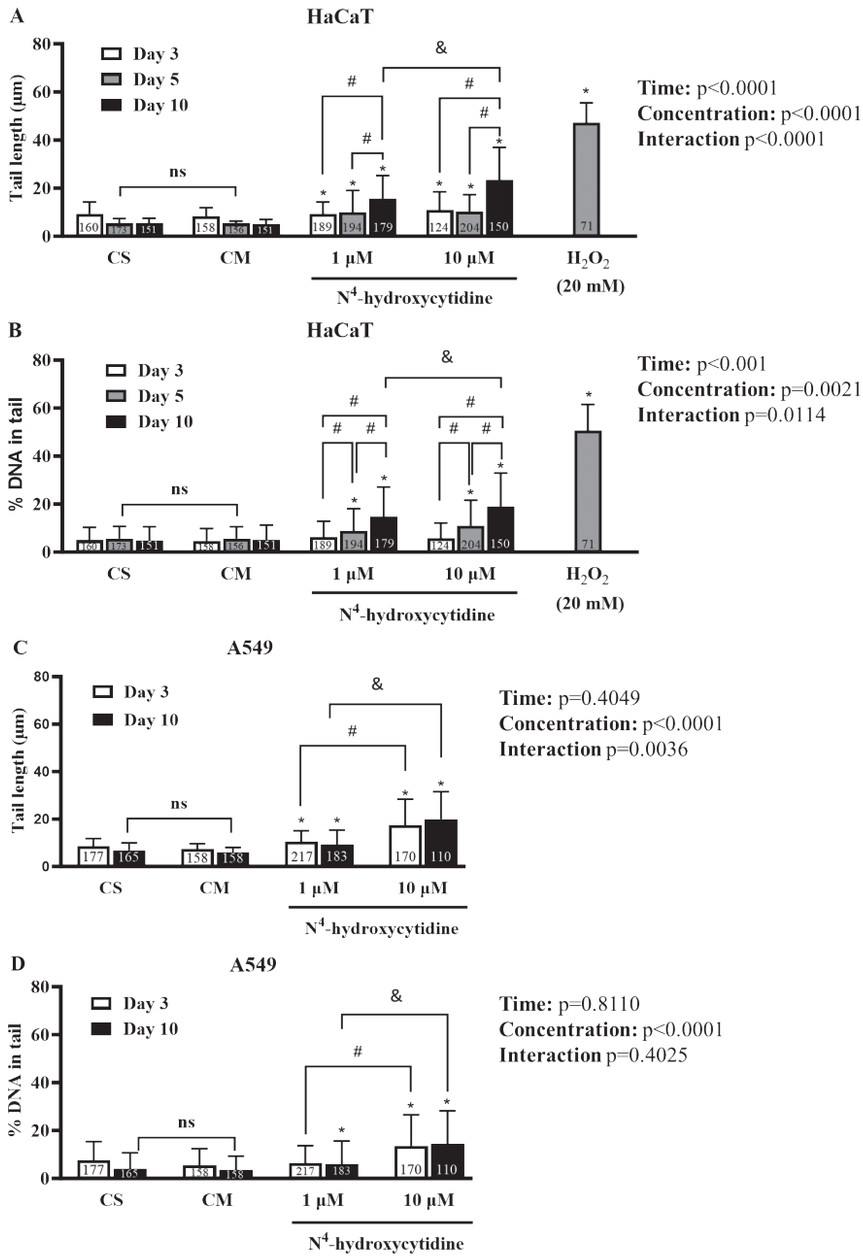

Figure 4 Comet assay analysis of DNA damage in response to N^4-hydroxycytidine exposure in HaCaT (A-B) and A549 (C-D) cells. DNA breaks were quantified in DNA tail length and % DNA in tail. A positive control was demonstrated by exposing day-5 cultured cells with hydrogen peroxide (A-B). P-values in (A-D) were analyzed by two-way ANOVA. *significant differences compared to CM at similar exposure time, analyzed by using unpaired t-test; #significant differences among different NHC concentrations (0 in CM, 1, and 10 µM) at similar exposure time, analyzed by using one-way ANOVA with Tukey's multiple comparison tests; &significant differences when comparing groups with different exposure durations and different concentration treatments, analyzed by using two-way ANOVA with Sidak's multiple comparison test. Data are presented as mean ± SD. The numbers on each bar indicates the number of cells in duplicate experiments. CM: control media; CS: control solvent (DMSO 0.3% v/v); ns: not significant.

Differential DNA damage after NHC exposure of HaCaT vs A549 cells

At 1μM NHC exposure for 3 days, DNA tail length in HaCaT was greater than A549 cells (9.28 ± 4.97 vs 10.57 ± 4.52μm, respectively, p = 0.0064), but %DNA in tail was similar in the two groups (6.31 ± 6.58 *vs* 6.38 ± 7.32μm, respectively, p>0.05). At longer duration of 1μM NHC exposure for 10 days, both tail length and %DNA in tail of HaCaT were greater compared to A549 cells (15.74 ± 9.48 *vs* 9.21 ± 6.13μm, p<0.0001, and 14.79 ± 12.35 *vs* 5.87 ± 9.76, p<0.001, respectively). Conversely, at 10 μM NHC exposure for 3 days, DNA tail length and %DNA damage were greater in A549 compared to HaCaT cells (17.45 ± 10.95 vs 10.92 ± 7.58, p<0.0001, and 13.61 ± 12.97 *vs* 5.74 ± 6.35, p<0.0001, respectively). At longer exposure of 10μM NHC exposure for 10 days, DNA tail length and %DNA damage of HaCaT were greater than A549 cells (2.47 ± 13.50 vs 19.91 ± 11.59, p = 0.0265, and 19.09 ± 13.97 vs 14.53 ± 13.69, p = 0.0086, respectively). The comparison analysis of comet assay between the two cell types was summarized in Table 2.

Table 2 Comparison of comet assay parameters between HaCaT and A549 cells after NHC exposure

Exposure conditions		Cell lines	N	Tail length (μm)		%DNA in tail	
Conc.	Time (days)			Mean ± SD	*P*-value	Mean ± SD	*P*-value
1 μM NHC	3	HaCaT	189	9.28 ± 4.97	0.0064*	6.31 ± 6.58	ns
	3	A549	217	10.57 ± 4.52		6.38 ± 7.32	
	10	HaCaT	179	15.74 ± 9.48	<0.0001*	14.79 ± 12.35	<0.0001*
	10	A549	183	9.21 ± 6.13		5.87 ± 9.76	
10 μM NHC	3	HaCaT	124	10.92 ± 7.58	<0.0001*	5.74 ± 6.35	<0.0001*
	3	A549	170	17.45 ± 10.95		13.61 ± 12.97	
	10	HaCaT	150	23.47 ± 13.50	0.0265*	19.09 ± 13.97	0.0086*
	10	A549	110	19.91 ± 11.59		14.53 ± 13.69	

* significant differences between the two cell types at similar concentration and exposure duration (unpaired t-test). Conc: concentration; N: the number of cells; NHC: N4-hydroxycytidine; ns: not significant.

Discussion

NHC induces cytotoxicity in vitro

European Medicines Agency reported *in vitro* data showing that NHC exerted antiviral activity to SARS-CoV-2 in various cell types including HUH-7, Calu-3, and A549-ACE2 with EC_{50} values in μM range. NHC antiviral activity is not due to cellular toxicity since its 50% cytotoxic concentration values (CC_{50}) were above the effective concentration values (EC_{50}).[24] Molnupiravir is rapidly hydrolyzed by carboxylesterases to NHC prior to reaching the systemic circulation.[25] NHC does not bind to plasma proteins.[25] Painter and coworkers reported that NHC was detected rapidly in the systemic circulation. The time to maximum plasma concentration (T_{max}) of NHC is ranged between 1-2 hours after a single oral dose of 800-1,600 mg molnupiravir. A single oral dose of 800, 1,200, and 1,600 mg results in maximum plasma concentration (C_{max}) of NHC at 3,640 ng/mL (14.04 μM), 4,500 ng/mL (17.36 μM), and 6,350 ng/mL (24.50 μM), respectively.[26] The NHC plasma molarity was calculated by using molecular weight of NHC at 259.22 Daltons.[27] Therapeutic dose of 800 mg molnupiravir every 12 hours results in NHC plasma concentration at steady state of 2,970 ng/mL (11.46 μM).[25] The human plasma concentrations of NHC detected in 1-10 μM range are relevant to the NHC IC_{50} values obtained from HaCaT cells *in vitro*. NHC-induced cytotoxic activity was more potent in HaCaT cells compared to human A549 cells. Less responsive NHC-induced cytotoxicity in A549 cells could be due to drug resistance characteristics of cancer cells. This finding is relevant to the work of Wallace and colleagues showing similar threshold of cytotoxicity by using SRB assay in the liver can-

cer cell HepG2 after 48 hours of NHC exposure.[28] Moreover, NHC-induced cytotoxicity in HaCaT human keratinocytes could be partly accountable for the NHC-related skin adverse events reported in patients with molnupiravir treatment.[29,30] Although a phase 2a clinical trial reported that low adverse events were associated with molnupiravir treatment (200-800 mg twice daily for 5 days),[31] this trial did not include participants with risk factors. Law and coworkers reported liver enzyme elevation after molnupiravir treatment in humans, and proposed caution when used in patients with impaired hepatic function.[32] Further assessment of NHC-induced cytotoxicity in other normal cell lines associated with vital organs is needed.

NHC induces DNA damage *in vitro*

Comet assay is a gel electrophoresis technique for detection of single cell DNA breaks.[33] As previously published data has reported NHC-induced mutagenesis in mammalian cells,[9] the current study aimed to determine whether NHC exposure caused phenotypic damage to DNA *in vitro*. The rapidly growing HaCaT and A549 cells were chosen for this study. Increases in the tail length and %DNA in tail were common parameters used for quantification of DNA breaks.[19] Our data suggested that DNA damage occurred in both cell types after exposure to 1-10 μM NHC for 3-10 days. NHC-induced DNA damage in HaCaT cells was concentration-dependent and time-dependent, whereas the effect in A549 was concentration-dependent, but not time-dependent. The resistance of NHC-induced DNA damage after a period of time was observed in A549 cells. This could be partly due to development of acquired resistance found in A549 cells.[34] Related mechanisms of this resistance include: overexpression of drug target genes[35] and multi-drug resistant genes[36], generation of tumor microenvironment,[34,35] and epithelial to mesenchymal transition.[34] Wallence group did not observe changes in mitochondrial gene expression in HepG2 cancer cells after 48 hours of NHC exposure. On the other hand, Miranda and coworkers showed that NHC caused an increase in genome-wide mutation frequencies in a concentration-dependent manner in mouse lymphoma L5178Y cells, and human lymphoblastoid TK6 cells.[37] Bian and colleagues suggested the antiviral drug prophylaxis after high-risk exposure for vulnerable cancer patients to prevent severe COVID-19.[38] Taken together, molnupiravir might not be a suitable candidate in this case, due to the possibility of drug resistance. Moreover, our findings were consistent with the data from Kobayahi et al., which revealed that NHC induced oxidative damage to isolated DNA from calf thymus was associated with the generation of NHC-induced hydroxylamine and Copper(I)-hydroperoxo complex generation.[39] Additional study of NHC-induced DNA damage and mutations in other normal cell lines associated with vital organs is suggested.

In conclusion, even though the safety profile of molnupiravir is still controversial, the study findings on cytotoxic and genotoxic activity of its active xenobiotic NHC, provide valuable information for the evaluation of the benefit/risk profile for molnupiravir. With the limitations of our *in vitro* study, we suggest that molnupiravir should be used with caution and supervision, especially in patients with impaired xenobiotic clearance. Furthermore, patients with cancer may be less responsive to molnupiravir treatment. In order to fulfill the safety profile of molnupiravir, the assessment of NHC-induced cytotoxicity and genotoxicity should be further performed in other normal cell lines associated with vital organs.

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Compliance with Ethics Requirements Experimental procedures and protocols were reviewed and approved by Institutional Biosafety Committee (IBC), Thammasat University (Approval number: 085/2565; Project code: 084/2565).

Conflict of interest All authors report no conflict of interest relevant to this article.

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Author Contributions

Participated in research design: Chamod, Sangsiri, Prajuabjinda, Poomirat, Tangjittham

Conducted experiments: Chamod, Poomirat, Prajuabkinda, Tangjittham, Liu, Mongkhonsakunrit, Pakotiprapha, Rimdusit

Performed data analysis: Chamod, Sangsiri, Rimdusit

Wrote or contributed to the writing of the manuscript: Chamod, Poomirat, Prajuabjinda, Sangsiri

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Original Article

Potential *in vitro* Anti-inflammatory and Anti-oxidant Activities of Various Extracts of *Etlingera elatior* Inflorescences

Weerachai Pipatrattanaseree[1], Thitiporn Thaptimthong[2], Narumon Boonrasri[3], Sadudee Rattanajarasroj[2], Sakwichai Ontong[2], Siriwan Chaisomboonpan[2]

Abstract

Introduction: *Etlingera elatior* (Jack) Smith has been used as folk medicine for earaches and wound cleansing. Although some parts of the plant have been studied for anti-inflammatory properties, there is a lack of research on the inhibition of nitric oxide (NO), prostaglandin E_2 (PGE_2), and tumor necrosis factor α (TNF-α). The findings of this study demonstrated its anti-inflammatory effects and suggested an effective extraction method for further investigations.

Objectives: This study aimed to explore the anti-inflammatory and antioxidant properties of *E. elatior* inflorescence extracts.

Methods: The bracts of *E. elatior* inflorescences were dried and separately extracted by 95%, 70%, and 50% ethanol maceration and decoction. The anti-inflammatory activity was investigated by inhibiting the production of NO, PGE_2 and TNF-α in RAW264.7 cells. Additionally, the anti-oxidant activity was assessed by DPPH scavenging assay.

Results: The 70% ethanolic extract of *E. elatior* exhibited both inhibitory effect on NO production (IC_{50} = 16.36 µg/mL) and DPPH scavenging activity (EC_{50} = 23.78 µg/mL), whereas the 95% ethanolic extract showed comparable inhibitory activity on NO (IC_{50} = 16.78 µg/mL) but not in antioxidant activity. The 95% ethanolic extract also showed moderate inhibition of PGE_2 production (IC_{50} = 45.26 µg/mL).

Conclusion: The ethanolic extracts of *E. elatior* inflorescences, obtained through maceration with 70% ethanol exhibited remarkable anti-inflammatory and anti-oxidant properties. 95% ethanol extracts exhibited anti-inflammation by both inhibition of NO and PGE_2. Consequently, it is imperative to further explore the 70% and 95% ethanolic extracts through in-depth research, including *in vivo* studies, phytochemical analysis, and anti-inflammatory product development.

Keywords: *Etlingera elatior*, Torch ginger flowers, Anti-inflammation, Anti-oxidation, Nitric oxide

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[1] Regional Medical Science Center 12 Songkhla, Department of Medical Sciences, Ministry of Public Health, Songkhla 90100, Thailand
[2] Medicinal Plant Research Institute, Department of Medical Sciences, Ministry of Public Health, Nonthaburi 11000, Thailand
[3] Veterinary Research and Development Center (Lower Southern Region), Department of Livestock, Ministry of Agriculture and Cooperatives, Songkhla 90110, Thailand
***Corresponding author:** Weerachai Pipatrattanaseree, Regional Medical Science Center 12 Songkhla, Department of Medical Science, Ministry of Public Health, Songkhla 90100, Thailand, Email: weerachai.tu2557@gmail.com

Introduction

Inflammation is a complex biological response that protects the body against harmful stimuli[1]: including infections, chemical exposure, tissue damage, and exposure to bacterial components like lipopolysaccharide (LPS).[2,3] Various cells and mediators, such as neutrophils, mast cells, and macrophages, play crucial roles in the inflammatory process, which produce proinflammatory cytokines and mediators, like nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), and prostaglandin E_2 (PGE_2), during inflammation.[4] While these mediators and cells aid in protecting the body, they can also affect normal cells and tissues with the excessive activation leading to cell and tissue damages resulting in inflammatory conditions such as pain, arthritis, including rheumatoid arthritis, diabetes, inflammatory bowel disease, atherosclerosis, and cancer.[5] The management of inflammation and pain typically involves the utilization of non-steroidal anti-inflammatory drugs (NSAIDs) and steroidal medications. Nevertheless, prolonged usage of steroids and NSAIDs can give rise to adverse effects like bleeding, indigestion, cardiac problems, and kidney toxicity.[6] Consequently, certain patients necessitate alternative medications such as herbal or traditional remedies. Hence, there is a growing inclination towards exploring substances derived from natural sources.

Etlingera elatior (Jack) Smith, commonly known as torch ginger, is a plant belonging to the Zingiberaceae family. It is extensively cultivated in Southeast Asia and other tropical regions, including Thailand, Malaysia, Indonesia, and the Boneo Peninsula.[7-9] It is named as "Da-Lah" in Thailand and is consumed as food ingredient or juice. In Malaysian folk medicine, the raw inflorescence is utilized to treat earaches and cleanse wounds[9]. Previous studies have reported various pharmacological activities of *E. elatior*, such as antioxidant properties[10-12], antibacterial effects[13], cytotoxic activity[11], anti-inflammatory properties[14], and anti-aging effects.[15,16] However, these studies mostly report on the rhizome and leaf of this plant. The analysis of phytochemicals in *E. elatior* inflorescences revealed the presence of flavonoids, terpenoids, saponins, and tannins.[10] Furthermore, gallic acid, tannic acid, chlorogenic acid, caffeic acid, quercetin, apigenin, kaempferol, luteolin and myricetin were identified as its chemical constituents.[13] These compounds showed both antioxidant and anti-inflammatory activities.[17] With regard to anti-inflammation, a study conducted on Wistar rats with induced gastric ulceration revealed the anti-inflammatory activity of *E. elatior* flower extract through the modulation of nuclear factor-kappaB-p65 (NF-κB-p65).[14] Another study by Aldi et al. demonstrated the immunomodulatory effects of *E. elatior* extract in allergic male white mice.[18] Nurhayatun and colleagues reported that the methanol extract of the fruit exhibited anti-inflammatory properties by reducing the levels of NF-κB, caspase 3, and IL-1ß in a Mus musculus sepsis model[19]. These findings indicate that the extract derived from various parts of *E. elatior* possesses anti-inflammatory activity. However, there is a lack of research on the inhibition of NO, PGE_2, and TNF-α production of the inflorescences. Therefore, the objective of this study is to explore the anti-inflammatory activity by inhibiting the production of NO, PGE_2, and TNF-α in RAW264.7 cells. Additionally, we evaluated the antioxidant properties using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay. This study not only provides information about the anti-inflammatory effects of *E. elatior* inflorescences extract but also demonstrates an effective extraction method for a further comprehensive investigation of its anti-inflammatory activity, quality control of the extract, and product development.

Methods

Plant materials and preparation of extracts

E. elatior was collected from Yala province, Thailand, in December 2020 and authenticated by Mr. Sakwichai Onthong, the Medical Plant Research Institute, Department of Medical Science, Thailand. The plant specimen (voucher number DMSC 5256) was deposited at the herbarium of Medicinal Plant Research Institute, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand.

The inflorescences of *E. elatior* were washed and the floral bract, involucral bract, and true flower were pulled out from the receptacle. The bracts were combined and subjected to drying in a hot air oven at a temperature of 45 ºC. The dried crude was then separately extracted through maceration and decoction methods. For maceration, the crude was separately macerated with ethyl

acetate, 95% ethanol, 70% ethanol, and 50% ethanol (3 days x 3 times). Each extract was filtered and evaporated under reduced pressure at a temperature below 45ºC. For decoction, the crude was boiled in distilled water for 15 minutes, followed by filtration using filter paper. The residue underwent two additional extractions using the same procedure, and the resulting extracts were combined and dried using a freeze dryer to obtain the aqueous extract. All samples were stored at -20 ºC until used.

Chemicals and reagents

Murine macrophage cell line (RAW 264.7: ATCC® TIB-71™) were purchased from American Type Culture Collection (ATCC®, VA, USA). Fetal bovine serum (FBS) was purchased from HyClone Laboratories, Inc (UT, USA). Dulbecco's Modified Eagle's Medium (DMEM), penicillin-streptomycin (P/S), phosphate buffer saline (PBS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2*H*-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (MO, USA). Prostaglandin E_2 and Tumor necrosis factor-a immunoassay kits were purchased from Enzo Life Sciences, Inc. (NY, USA).

Cell culture

RAW 264.7 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 µg/L streptomycin, and 100 IU/mL penicillin at 37 °C with 95% humidity in a 5% CO_2 atmosphere.

Cell viability assay

Cell viability was measured by the mitochondria-dependent reduction of MTT (3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution to formazan. RAW 264.7 cells were seeded in 48-well plates at the density of 1.2 x 10^5 cells per well and then cultured for 24 hours. The cells were treated with various concentrations of *E. elatior* extracts (1-100 κg/mL) for 24 hours. Subsequently, cells were incubated with MTT solution at final concentration of 0.1 mg/mL for 1 h at 37 °C. The medium was then removed and the formazan crystals were dissolved in DMSO. The absorbance was measured at 570 nm with correction at 650 nm using microplate reader (Biotek, VT, USA). The percentage of cell viability was calculated by comparing the treated cell to the control (100%). The cell viability of more than 85% indicated that the inhibitory effect was not resulting from cell death.

Measurement of nitric oxide

The determination of anti-inflammatory activity by inhibiting the NO production followed the method outlined in the study conducted by Miranda *et al.*[20] The cells were seeded into 48-well plate at a density of 1.2 x 10^5 cells per well and cultured for 24 hours. Subsequently, the cells were treated with various concentrations of *E. elatior* extracts (1-100 µg/mL). N omega-Nitro-L-arginine methyl ester hydrochloride (L-NAME; NOS inhibitor) was used as positive control. After 1 hour treatment, the cells were stimulated with 0.2 µg/mL of LPS and cultured for 24 hours. Nitric oxide production was determined by measuring the concentration of nitrite in culture medium using Griess's reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylene-diamine-HCl in water). Briefly, the culture medium in each well (100 µL) was transferred to another 96-well plate and then 100 µL of Griess's reagent was added and incubated for 10 minutes. The absorbance was measured at 520 nm, with correction at 665 nm. Nitrite concentrations were calculated from sodium nitrite ($NaNO_2$) standard curve. The inhibitory activity (%) of nitric oxide production was calculated by the following equation:

$$\text{Inhibition (\%)} = \left[\frac{N_{control} - N_{sample}}{N_{control}} \right] \times 100$$

Where $N_{control}$ was the nitrite concentration of the control and N_{sample} was the nitrite concentration of sample. The IC_{50} values were calculated from the GraphPad Prism software (GraphPad®, USA).

Measurement of PGE_2 and TNF-α

The cells were seeded into 48-well plate (1.2 x 10^5 cells per well) and cultured for 24 hours. The cells were treated with various concentrations of *E. elatior* extracts (1-100 µg/mL). Indomethacin was used as positive control of PGE_2 assay, whereas, dexamethasone was used for TNF-α assay. After 1 hour treatment, cells were stimulated by 0.2 µg/mL of LPS and cultured for 24 hours. The culture medium in each well was collected and kept in -80 ºC until being tested for PGE_2 and TNF-α. The quantity of PGE_2 and TNF-α in the cultured medium

were quantified by EIA kits according to the manufacturer's instructions (Enzo Life Sciences, USA). The inhibition (%) of PGE_2 and TNF-α production was calculated by the following equation:

$$\% \text{ Inhibition} = \left[\frac{OD_{control} - OD_{sample}}{OD_{control}} \right] \times 100$$

Where $OD_{control}$ was the absorbance of the control and OD_{sample} was the absorbance of sample. The IC_{50} values were calculated from the GraphPad Prism software (GraphPad®, USA).

DPPH Radical Scavenging Activity

DPPH scavenging activity as a model of antioxidant activity was conducted following the modified method of Yamasaki et al.[21] The extract was dissolved in absolute ethanol or distilled water to achieve a concentration of 2000 µg/ml as a sample stock solution. Subsequently, each extract was further diluted to obtain working solution at concentrations of 200, 100, 60, 20, and 2 µg/ml. The DPPH scavenging reaction started by adding 100 µl of $6x10^{-5}$ M DPPH in 100 µl aliquot of the working solution and stored in darkness at room temperature for 30 minutes. The absorbance was subsequently measured at 520 nm. Butylated hydroxytoluene (BHT) was used as a positive control. Inhibition (%) was calculated using the following equation.

$$\% \text{ Inhibition} = \left[\frac{OD_{control} - OD_{sample}}{OD_{control}} \right] \times 100$$

Where $OD_{control}$ was the absorbance of the control and OD_{sample} was the absorbance of sample. The IC_{50} values were calculated from the GraphPad Prism software (GraphPad®, USA).

Statistical analysis

The experiments were performed in triplicate, and the results were presented as the mean ± standard deviation (SD). The EC_{50} and IC_{50} values were determined by regression analysis, and for multiple comparisons of dataset, ANOVA and Dunnett's post-hoc test was conducted. All statistical analyses were calculated by using GraphPad Prism software (GraphPad®, USA).

Results

Plant material extractions

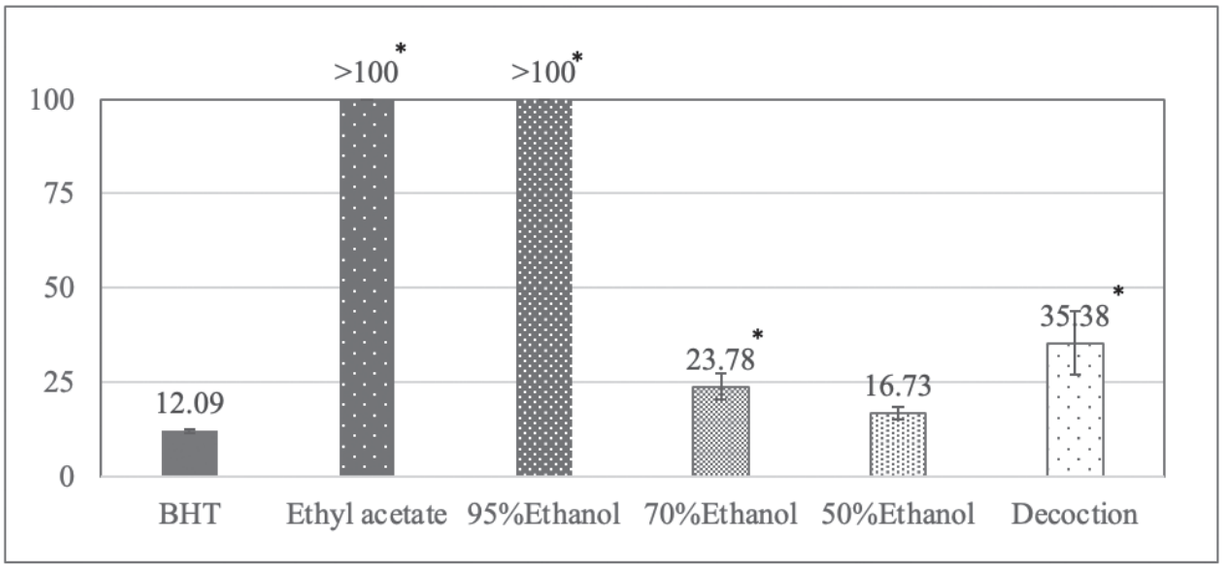
The percentage yields of the plant extracts are shown in Table 1. The extraction yield obtained from 95% ethanol maceration demonstrated the highest value of 19.96%, followed by the 70% ethanol extract. The ethyl acetate extract demonstrated the lowest yield, with a value of 9.82%.

Table 1 Yield (%) of the extracts obtained from various extraction method

Extraction solvent	Extraction method	Code	Yield (%)
Ethyl acetate	Maceration	EEE	9.82
95% Ethanol	Maceration	EE95	19.96
70% Ethanol	Maceration	EE70	18.93
50% Ethanol	Maceration	EE50	16.66
Water	Decoction	EEDec	15.76

DPPH scavenging activity

As shown in Figure 1, the 50% ethanolic extract (EE50) exhibited the highest DPPH scavenging activity, with an EC_{50} value of 16.73 ± 1.58 µg/mL, which was not significantly different from BHT (12.09 ± 0.50 µg/mL). The EEE and EE95 demonstrated no activity in DPPH scavenging (EC_{50} > 100 µg/mL). The EE70 and EEDec showed moderate DPPH scavenging activity, with EC_{50} values of 25.12 ± 2.88 µg/mL and 35.38 ± 8.35 µg/mL, respectively, which were significantly higher than that of BHT.



*Significant different from the positive control, BHT, analyzed by ANOVA with Dunnett's analysis (p-value<0.05)

Figure 1 DPPH scavenging activity of *E. elatior* extracts obtained from various extraction solvent.

Inhibitory effects on nitric oxide production

The inhibitory activity of *E. elatior* extracts on the production of nitric oxide from the *LPS*-induced RAW264.7 cells are showed in Table 2. Among the extracts tested, EE70 exhibited the most potent inhibitory effect on nitric oxide production, with an IC_{50} value of 16.36 ± 4.12 µg/mL. EE95 and EEE extracts exhibited IC_{50} values of 16.78 ± 7.21 and 22.59 ± 3.33 µg/mL, respectively. Both EE50 and EEDec demonstrated no activity (IC_{50} > 100 µg/mL). However, all extracts possessed inhibitory effect less than the NOS inhibitor, L-NAME. Moreover, the extracts did not exhibit cytotoxic activity, as showed by the percentage survival exceeding 80%. This indicates that the inhibitory activity of the extracts is not a result of cell death.

Table 2 Inhibitory effects of *E. elatior* extracts on nitric oxide, PGE_2 and TNF-α production from RAW 264.7 cells.

Sample	Inhibitory effects (IC_{50}; µg/mL)		
	Nitric oxide	PGE_2	TNF-α
EEE	22.59 ± 3.33*	66.56 ± 14.59*	>100*
EE95	16.78 ± 7.21*	45.26 ± 9.28*	>100*
EE70	16.36 ± 4.19*	>100*	>100*
EE50	96.09 ± 12.1*	>100*	>100*
EEDec	>100*	>100*	>100*
L-NAME	6.69 ± 1.95 (0.029 ± 0.008 µM)	NA	NA
Indomethacin	NA	9.31 ± 3.23 (0.026 ± 0.009 µM)	NA
Dexamethasone	NA	NA	35.73 ± 15.31 (0.091 ± 0.039 µM)

*Significant difference from the positive control (*p*-value <0.05) analyzed by ANOVA with Dunnett post-hoc analysis.

Inhibitory effects on PGE_2 and TNF-α production

As shown in Table 2, EE95 and EEE are only two extracts exhibiting the production of PGE_2 of the *LPS*-induced RAW264.7 cells with IC_{50} values of 45.26 ± 9.28 and 66.56 ± 14.59 µg/mL, respectively, other extracts possessed no inhibitory activity. The positive control, indomethacin showed potent inhibitory effect significantly more than the extracts (IC_{50} = 9.31 ± 3.23 µg/mL; 0.026 ± 0.009 µM). With regard to inhibitory effect on TNF-α production, none of the extracts exhibited inhibitory activity, while the positive drug, dexamethasone, exhibited potent activity with IC_{50} value of 35.73 ± 15.31 µg/mL (0.091 ± 0.039 µM).

Discussion

E. elatior is an edible plant which commonly consumed in south-east Asia particularly in Thailand and Malaysia. It has been used as a folk medicine in Malaysia for the treatment of earache and as a wound cleaner[9]. This study aimed to investigate anti-inflammatory activity by inhibiting NO, PGE_2 and TNF- α production in RAW264.7 cells. Additionally, the anti-oxidant by DPPH scavenging assay were also evaluated.

For anti-oxidant activity, our study revealed that the 50% ethanolic extract exhibited potent antioxidant properties as demonstrated by the DPPH scavenging assay, which were comparable to the positive control, BHT. A previous study conducted by Lachumy and colleagues also reported potent DPPH scavenging activity comparable to BHT for the 80% methanolic extract[12]. These findings support the result that *E. elatior* flower possesses DPPH scavenging activity, thus suggesting its potential utilization as an anti-oxidant product.

With regard to the inflammatory activity, *E. elatior* extract demonstrated the most inhibitory effect on NO production in the induced RAW264.7 cell. Interestingly, the EE70 and EE95 showed potent inhibition with IC_{50} values less than 20 µg/mL. EE95 also inhibited the PGE_2 production. The results of our study related to a previous study of Juwita and colleague, which demonstrated that the 95% ethanolic extract obtained through the soxhlet method effectively suppressed the development of gastric ulceration by down-regulating the expression of NF-kB-p65[14]. These results indicate that *E. elatior* 95% ethanolic extract possessed inhibitory effect on nitric oxide. Moreover, our study showed that the EE70 demonstrated potent NO inhibition as well as the EE95 and EEE extracts. In contrast, the aqueous extract and 50% ethanol extract displayed no effects in all anti-inflammatory activities. These findings suggest that compounds found in *E. elatior*, which were more likely to dissolve in ethanol, tend to inhibit the production of NO and PGE_2. Nevertheless, it is important to note that the phytochemical analysis conducted in this study had limitations, and further research is needed to explore this aspect comprehensively, including assessments of total phenolics, total flavonoids and chemical constituents.

Nitric oxide (NO) plays a dual role intrinsically. It serves as a vital physiological signaling molecule, facilitating diverse cellular functions. However, in contrast, it elicits cytotoxic and mutagenic effects when present in abundance, particularly under conditions of oxidative stress. It plays a significant role in the prolongation of inflammation and immunological responses[22]. Excessive NO production from inflammatory cells lead to several diseases by several mechanisms. As a pro-inflammatory cytokine NO can be produced by various cell types, including immune cells such as macrophages and neutrophils, during chronic inflammation. It can contribute to the inflammatory response through several mechanisms particularly by the induction of cytokines. NO can stimulate the production of other pro-inflammatory cytokines, including interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6). These cytokines contribute to the amplification of the inflammatory response[23]. Therefore, inhibiting the excessive production of NO can help reduce these inflammatory responses.

In conclusion, our study presents the initial findings that the ethanolic extracts of *E. elatior* inflorescences, obtained through maceration with 70% ethanol and 95% ethanol, exhibited remarkable anti-inflammatory activity by inhibiting the production of NO. In addition, EE95 exhibited moderate anti-inflammatory activity in suppressing the production of PGE_2 and the 70% ethanolic extract demonstrated high DPPH scavenging activity. Consequently, the 95% and 70% ethanolic extracts should be investigated in in-depth research, including *in vivo* studies, phytochemical analysis, and quality control of extract for further anti-inflammatory product development.

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Compliance with Ethics Requirements. Not applicable.

Conflict of interest. The authors declare that there is no conflict of interest regarding the publication of this paper.

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Author Contributions. Weerachai Pipatrattanaseree designed all experiments, collected the plant materials, prepared the plant material and extracts, performed anti-oxidant experiment and wrote the original draft manuscript. Thitiporn Thaptimthong conducted anti-inflammatory experiments and assisted in writing the original draft manuscript. Narumon Boonrasri helped preparation of extracts. Sadudee Rattanajarasroj advised and supported Thitiporn Thaptimthong for anti-inflammatory experiments. Sakwichai Onthong was responsible for identifying the plant specimen. Siriwan Chaisomboonpan provided valuable supported and commented throughout the entire project. All authors read and approved the final version of the manuscript.

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Original Article

The Correlations between Three Methods of Pelvic Floor Muscle Strength Assessment in Nulliparous Women: 2D Transperineal Ultrasound, Modified Oxford Scale, and PFX2® Perineometer

Puckjira Iaocharoen[1*], Orawan Lekskulchai[1], Keerati Chiengthong[2], Suvit Bunyavejchevin[2], Chamnan Tanprasertkul[3], Charintip Somprasit[4]

Abstract

Objective To study the correlation between three methods for pelvic floor muscle strength assessment in nulliparous women.

Methods: A cross-sectional study, 50 nulliparous were recruited. Modified oxford scale (MOS) was assessed by one author (OW) and highest maximum squeeze value was recorded. The vaginal pressure during maximum squeeze with PFX2® perineometer was recorded by one trained nurse. The midsagittal view of anteroposterior (AP) hiatal dimension using 2D transperineal ultrasound (TPUS) was done by the other author (PL) to measure the difference between the AP hiatal dimension in the resting stage compared to maximum squeeze.

Results: The mean MOS ± SD was 4.4 ± 0.7. The mean ± SD PFX2® perineometer was 10.4 ± 1.8 cmH2O. The mean ± SD difference of AP dimension using TPUS was 1.1 ± 0.6 cm (22.8 ± 10%). PFX2® perineometer was poorly correlated with the different AP dimension using TPUS (r = 0.19, *p-value* = 0.18) and weakly correlated with the percent of difference AP dimension using TPUS (r = 0.21, *p-value* = 0.15). MOS was moderately correlated with the difference and percent of difference AP dimension using TPUS (r = 0.35, *p-value* <0.05 and r = 0.34, *p-value* <0.05 respectively). MOS was strongly correlated to PFX2® perineometer (r = 0.73, *p-value* < 0.05).

Conclusions: In healthy nulliparous women, PFX2® perineometer and MOS could be used to assess the strength of the pelvic floor muscles, but two-dimension TPUS could not be used to assess it. Because the difference hiatal dimension is small due to nulliparous characterization.

Keywords: Transperineal ultrasound, Perineometer, Pelvic floor muscle strenth

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[1] Urogynecologic clinic, Department of Obstetrics and Gynecology, Faculty of Medicine, Thammasat University, Pathum Thani, Thailand
[2] Female pelvic medicine clinic, Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand
[3] Center of excellence in applied epidemiology, Faculty of Medicine, Thammasat University, Pathum Thani, Thailand
[4] Department of Obstetrics and Gynecology, Faculty of Medicine, Thammasat University, Pathum Thani, Thailand
***Corresponding author:** Puckjira Iaocharoen M.D., Urogynecologic clinic, Department of Obstetrics and Gynecology, Faculty of Medicine, Thammasat University, Pathum Thani, Thailand, 12120. Phone: 02926-9343, Fax: 02-926-9485, E-mail: puckjiraaeocharoen@gmail.com

Introduction

Female pelvic floor muscles (PFM) consist of the levator ani (pubococcygeus muscle, iliococcygeus muscle, puborectalis muscle) and the coccygeus muscle. Their function is to support the pelvic structure including urethra, bladder, uterus and bowel. In case of PFM dysfunction, patients may present with pelvic organ prolapse, stress urinary incontinence, fecal incontinence, sexual dysfunction and chronic constipation among others.[1] The prevalence of pelvic organ prolapse is 3-50% (with a high prevalence in elderly woman). The prevalence of urinary incontinence is 25-45% (in Thailand, the prevalence is 36.5%).[2] These conditions may cause medical illness, distress and may affect the quality of life of women around the world.[2, 3]

Factors contributing to pelvic floor dysfunction include pregnancy, vaginal delivery, smoking, chronic cough, chronic constipation, and genetic factors.[4, 5] Vaginal childbirth may cause two types of levator ani muscle injury: macrotrauma (partial or complete tearing of muscle tissue) and microtrauma following the sudden distention of the levator ani muscle. Both injuries result in a decrease in the effectiveness of levator ani muscle contraction, a hiatal overdistention and a pelvic organ prolapse (especially uterine prolapse and cystocele).[6, 7]

The 6th International Consultation on Incontinence recommended that PFM training should be offered as a first line of treatment for urinary incontinence, fecal incontinence, and mild pelvic organ prolapse. PFM strength is the main focus for treatment in these patients. The success of PFM training depends on muscle strength, endurance and patients' diligence.[8, 9] There are several ways to assess PFM strength. Vaginal palpation by Modified Oxford Scale (MOS) can be used to assess PFM strength, but it could be qualitative and inaccurate. Because of this limitation, the perineometer (Peritron®, PFX2®) was invented to help assess PFM strength, but it is invasive and not widely available.[10] Transperineal ultrasound (TPUS) is the new method now used to assess PFM strength because it is non-invasive and easy to perform.[11]

In the past, studies comparing the three PFM strength assessment methods (MOS, perineometer, TPUS) in pelvic organ prolapse and urinary incontinence patients showed no statistically significant difference among different assessment methods.[12-14] In 2007, Nadia M, et al. reported a TPUS assessment of the PFM (morphological and dynamic function) in women with pelvic floor dysfunction symptoms. Their results showed that the mean percent of the difference in anteroposterior hiatal dimensions using TPUS (LHap) between rest and maximum squeeze decreased significantly with low MOS score for the women. A cut-off percent decrease in LHap between rest and maximum squeeze of less than 6.5% predicted a low MOS score.[15] At present, no comparative study among three PFM strength assessment methods (MOS, perineometer, and TPUS) in heathy nulliparous women has been undertaken.

The purpose of this study is to evaluate the correlation between the three methods of PFM strength assessment (TPUS, MOS, and PFX2® perineometer) in nulliparous women.

Materials and Methods

Study design

A cross-sectional study was conducted at Thammasat University Hospital (TUH), a tertiary referral center, in Thailand. The study obtained ethical approval from the TUH ethics committee. (MTU-EC-OB-0-240-63)

Study Population

Participants who were of the reproductive age (18-50 years old) from the general gynecology unit in Thammasat University Hospital were recruited. All participants signed an informed consent form.

Inclusion criteria were nulliparous, sexual active and able to contracted pelvic floor muscle correctly by visualization from TPUS examination. Exclusion criteria were women with pelvic organ prolapse, lower urinary tract symptoms (LUTs), history of gynecologic surgery, history of gynecologic cancer, prior pelvic reconstructive and anti-incontinence surgery, pregnancy, and inability to give informed consent.

Study Protocol

After signing the informed consent form, each participant answered a questionnaire concerning age, body weight, height, underlying disease, and family history of pelvic organ prolapse

and a Urogenital Stress Inventory questionnaire (UDI questionnaires) by a research nurse. All participants were examined by three blinded examiners. The sequence of the three PFM strength assessment methods was randomized to minimize measurement and information bias.

The participants began by making sure that their bladders were empty. They were then examined in the lithotomy position. A digital exam was performed and assessed by the co-author (OW) to assess pelvic muscle strength using the MOS (patients were told to squeeze 3 times and the highest value was recorded). The co-author inserted her index and middle fingers approximately 4 cm into the vagina canal of each patient and the puborectalis muscle at each side of the vagina was palpated during contraction. The pelvic floor muscle strength (by MOS) was classified as a scale of 0-5 (0 = no contraction; 1 = minor muscle 'flicker'; 2 = weak muscle contraction without a circular contraction; 3 = moderate muscle contraction; 4 = good muscle contraction and 5 = strong muscle contraction).[16]

PFX2® perineometer is a conical sensor covered with a medical silicone rubber sheath used for taking the vaginal pressure measurement. It was covered with a sterile latex sleeve for each patient and the middle of the balloon was placed approximately 3.5 cm inside the introitus. Patients were told to squeeze 3 times and the highest value was recorded by a trained nurse.

Two dimension TPUS using the SAMSUNG UGEOH60 B-mode capable 2D ultrasound system with cineloop function, a 3.5-6 MHz array transducer using a gel was performed by author (PL). The midsagittal view of genital hiatus was examined to measure the difference between the anteroposterior hiatal (AP) dimension in the resting stage and the maximum squeeze (the patients were instructed to squeeze three times and the highest value was recorded).[17-19] (Picture 1)

Statistical Analysis

A statistical analysis was performed with the STATA version 15. General characteristic was determined as the mean ± SD. Spearman's rank correlation coefficient (r) was used to assess the correlation among the three methods. The correlation was classified as r = 0-0.20, poorly correlated; r = 0.21-0.40, weakly correlated; r = 0.41-0.60, moderately correlated; r = 0.61-0.80, strongly correlated; r = 0.81-1, very strongly correlated.[20] A *p-value* < 0.05 was considered statistically significant.

Sample size

The sample sized was calculated by this formula[21]

$$\mathbf{N = [(Z_{\alpha} + Z_{\beta}) / c]^2 + 3}$$

$$N = [(1.96)^2+(1.282)^2/c^2] + 3$$

$$N= [(3.842+1.644)/ 0.116] + 3$$

$$N=47+3$$

$$N=50$$

N = The number of population

α = 5% significance level test = 0.05

β = probability of failing to reject the null hypothesis under the alternatiive hypothesis = 0.10

c = 0.5 x In [(1+r)/(1-r)] = 0.3416

r = the expected correlation coefficient from the pilot study = 0.329

Result

A total of 50 nulliparous women, all sexually active with no family history of pelvic organ prolapse, were included in study. The mean age ± SD of the participating women was 29 ± 7.9 years (minimal age was 20 years and maximum age was 40 years). They had a mean body weight ± SD of 53.7 ± 8.7 kg, A mean ± SD height was 161.5 ± 5.6 cm and a mean ± SD body mass index of 20.6 ± 3.1 kg/cm^2.

Their mean ± SD MOS was 4.4 ± 0.7 and their mean ± SD PFX2® perineometer was 10.4 ± 1.8. The mean ± SD of AP dimensions, using TPUS at rest and at maximum squeeze, were 4.9 ± 0.7 cm and 3.8 ± 0.6 cm respectively. The mean ± SD difference in their AP dimensions using TPUS was 1.1 ± 0.6 cm while the mean ± SD percent difference in their AP dimensions using TPUS was 22.8 ± 10 (Table 1)

According to the Spearman's rank correlation coefficient, the PFX2® perineometer was poorly correlated with the difference in AP dimensions using TPUS (r = 0.19, p-value = 0.18, as shown in Figure 1) and weakly correlated with the percent difference in AP dimensions using TPUS (r = 0.21, p-value = 0.15, as shown in Figure 2). Meanwhile, MOS correlated moderately with difference in AP dimensions using TPUS (r = 0.35,

p-value < 0.05, as shown in Figure 3) and with the percent difference in AP dimensions using TPUS (r = 0.34, p-value < 0.05, as shown in Figure 4). MOS was strongly correlated the PFX2® perineometer (r = 0.73, p-value < 0.05, as shown in Figure 5)

Table 1 The average result of three methods for pelvic floor muscle strength assessment.

Measurement method	Mean ± SD	Range
Modified oxford scale	4.4 ± 0.7	3-5
PFX2® perineometer (cmH_2O)	10.4 ± 1.8	6-12
AP*dimension using TPUS* *at rest (cm)	4.9 ± 0.7	3.3-6.4
AP*dimension using TPUS* *at maximum squeeze (cm)	3.8 ± 0.6	2.1-5.2
Mean difference of AP*dimension using TPUS** between rest and maximum squeeze (cm)	1.1 ± 0.6	0.1-3.2
Mean percentage difference of AP*dimension using TPUS**between rest and maximum squeeze (%)	22.8 ±10	2.6-51.6

*AP: Anteroposterior hiatal, **TPUS : Transperineal ultrasound

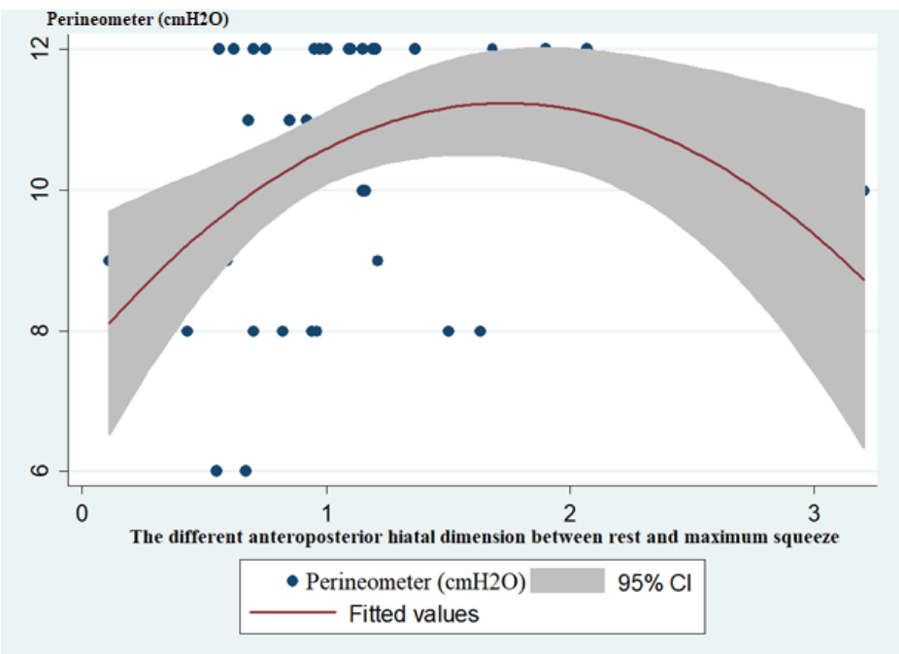

Figure 1 The Spearman's rank correlation coefficient between PFX2® perineometer and the difference anteroposterior hiatal dimension between rest and maximum squeeze (r = 0.19, p-value = 0.18)

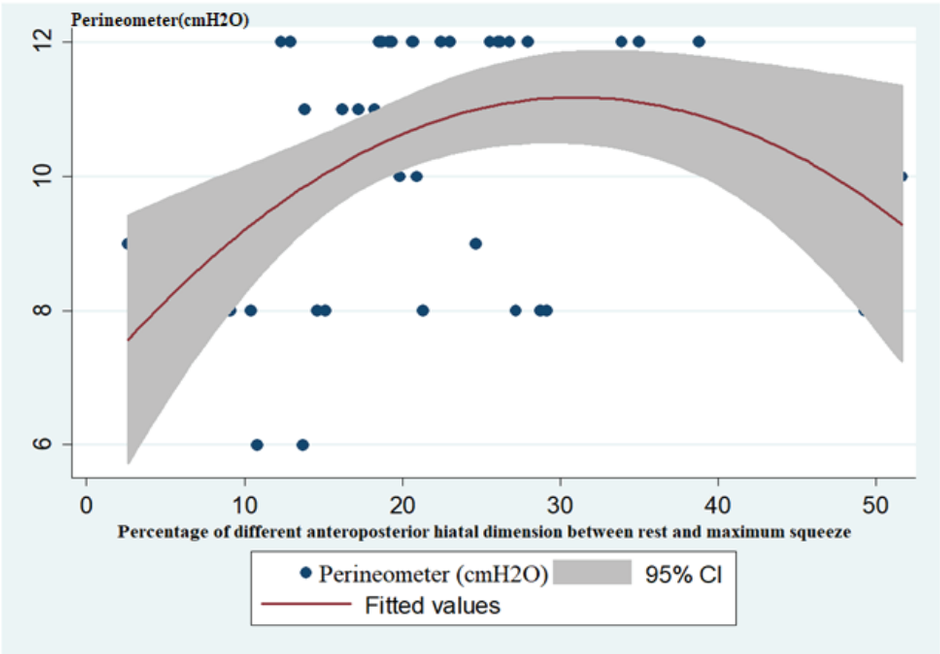

Figure 2 The Spearman's rank correlation coefficient between PFX2® perineometer and percent of difference anteroposterior hiatal dimension between rest and maximum squeeze (r = 0.21, p-value = 0.15)

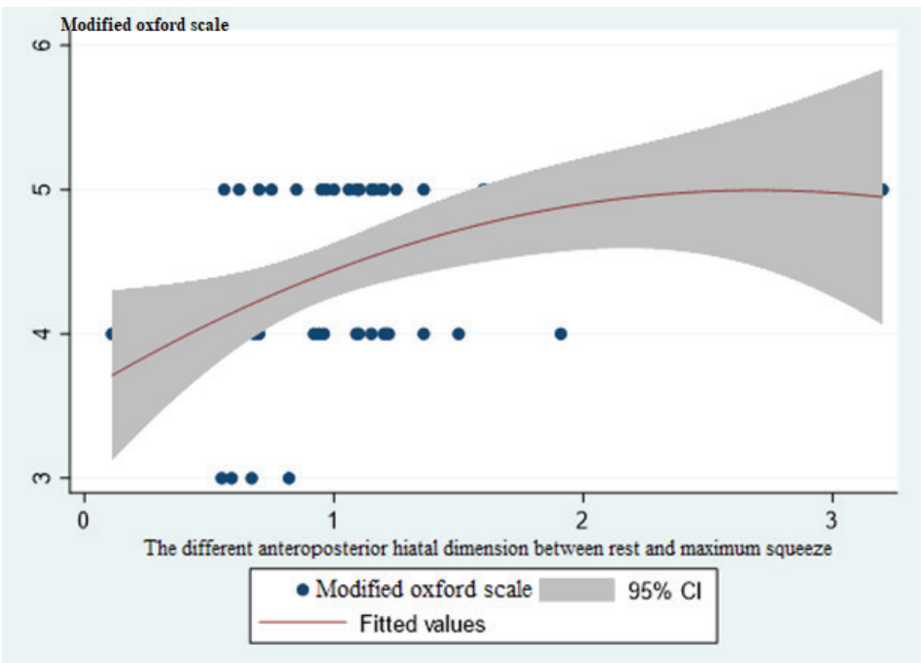

Figure 3 The Spearman's rank correlation coefficient between Modified Oxford scale and the difference anteroposterior hiatal dimension between rest and maximum squeeze (r = 0.35, p-value < 0.05)

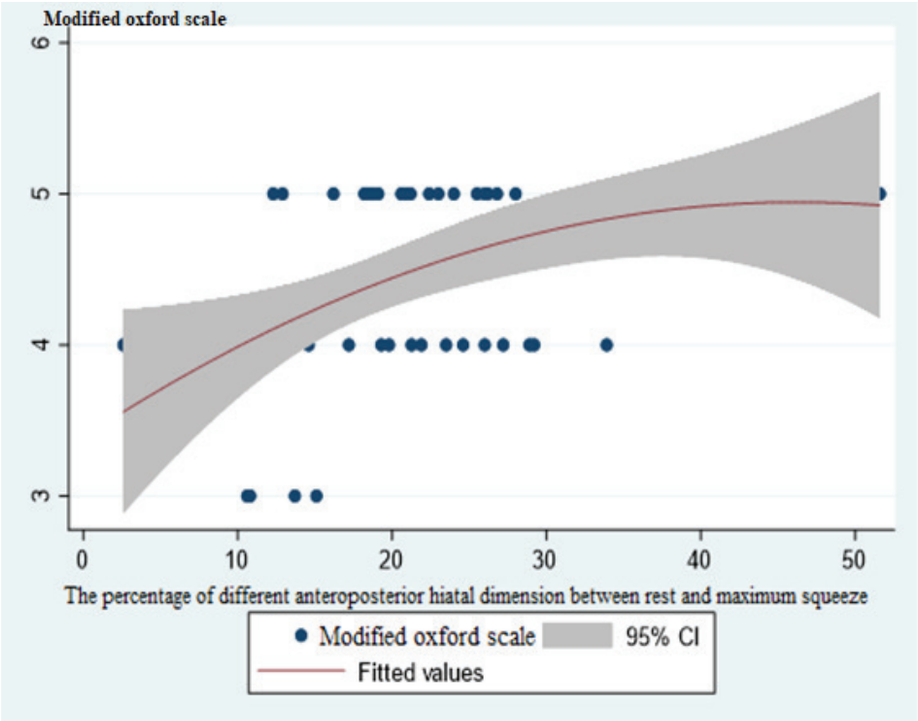

Figure 4 The Spearman's rank correlation coefficient between Modified Oxford scale (MOS) and percent of difference anteroposterior hiatal dimension between rest and maximum squeeze ($r = 0.34$, p-value < 0.05)

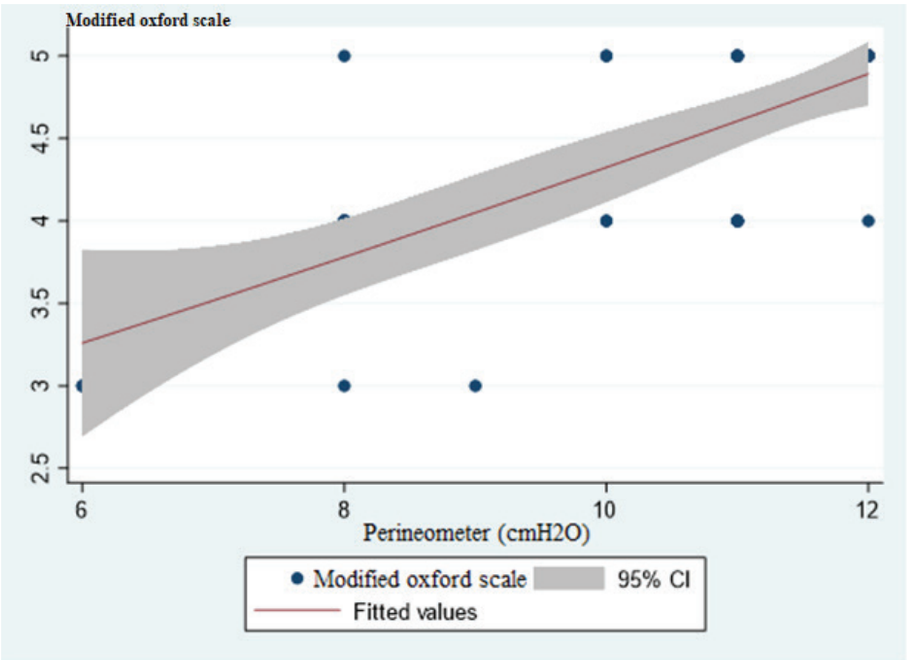

Figure 5 The Spearman's rank correlation coefficient between Modified Oxford scale (MOS) and PFX2® perineometer ($r = 0.73$, p-value < 0.05)

Discussion

The pelvic floor strength assessment is crucial for the diagnosis and treatment of patients with pelvic floor dysfunction, who show symptoms of pelvic organ prolapse, stress urinary incontinence, fecal incontinence, sexual dysfunction and chronic constipation. In most cases, pelvic floor dysfunction often follows vaginal birth and leads to injury of the PFM by causing tearing and stretching. In 2017, Nadia M, et al.[15] studied a TPUS assessment of the PFM (morphological and dynamic function) in women with pelvic floor dysfunction symptoms. The results showed that the mean percent difference in the AP dimensions using TPUS (LHap) between at rest and at maximum squeeze decreased significantly in low MOS women (r = 0.67). Thus, this study supports our hypothesis that widening the genital hiatus reduces PFM strength. There are various techniques to assess the strength of the pelvic floor, such as vaginal palpation using Modified Oxford scale (MOS), perineometer, 2D/3D/4D transperineal ultrasound, among others.

Our study showed that the difference in the AP dimensions using TPUS was poorly correlated with the PFX2® perineometer, which is the standard method of measurement (r = 0.19). The percent difference in the AP dimensions using TPUS weakly correlated with the PFX2® perineometer (r = 0.21), while both values of TPUS were moderately correlated with MOS (r = 0.35 and 0.34, respectively). Our results were different from the study conducted by VOLLØYHAUG, et al.[14] in 2015. They found that the correlation of change in hiatal AP diameter (at rest and maximum squeeze) with Camtech AS® perineometer was moderate (r = 0.58). However, our study has produced different results from that prior study, possibly because of the difference in demographics of the patients, especially multiparity, since multiparity is the most significant factor in incidences of pelvic floor dysfunction.

We noticed a small difference in AP dimension using TPUS at rest and maximum squeeze (the mean difference in the AP dimension using TPUS was 1.1 ± 0.6 cm and the mean percent difference in the AP dimensions using TPUS was 22.8 ± 10%) in the genital in our sample of nulliparous women. The nulliparous women experienced either minimal or no PFM trauma. They therefore had good muscular integrity.

The MOS measurement has weakness as a pelvic floor muscle strength measurement, because it is measured as a subjective value, it depends on the examiners experience for measuring vaginal pressure. This subjective measurement could be inaccurate. The perineometer (Peritron®, PFX2®) was developed to improve the level of accuracy by using quantitative measurements instead of qualitative assessments, as in the use of MOS, and is now the objective standard method. The disadvantage of the PFX2® perineometer is its invasiveness and the machine was designed for personal use. TPUS is a new objective method, which is noninvasive and easy-to-perform. It assesses PFM by measuring the difference in anteroposterior hiatal dimension, which is different from the two previously mentioned methods (MOS and perineometer), which measure the pressure of the vagina. Thus, this functional difference may be the reason that the correlation between the MOS and the perineometer was stronger, then when making comparisons by use of the TPUS.

TPUS measures the difference anteroposterior hiatal dimension, while the MOS and PFX2® perineometer measures the pelvic floor muscle power. We presumed that the difference in anteroposterior hiatal dimension was correlated to the pelvic floor muscle power. But from our study, we found poor correlation between TPUS and PFX2® perineometer. Using this parameter is not appropriate, because the difference anteroposterior hiatal dimension when resting and maximum squeezing is small due to nulliparous characterization.

The strength of this study was that it is the first prospective cross-sectional study to assess pelvic floor muscle strength by MOS, perineometer and different AP dimension using TPUS in healthy Asian nulliparous women of reproductive age. All participating women were clinically examined by three blinded examiners. Three PFM strength assessment methods were randomized to decrease measurement and informational bias. The examiners alone were blinded to the ultrasound-examination data, thus eliminating inter-observer variation, and data was randomized in each of the collection methods. The limitation of this study is that it is only a single center study and with one operator for its measurement. To confirm this negative result, the study should be repeated in other medical centers.

In healthy nulliparous women, PFX2® perineometer and MOS could be used to assess the strength of the pelvic floor muscles, but two dimensional TPUS should be avoided, because the difference in hiatal dimension is small in nulliparous women.

Financial support: -

Compliance with Ethics Requirements: none

Conflict of interest: none

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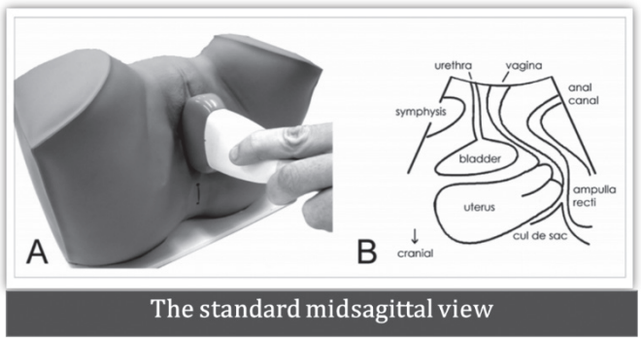
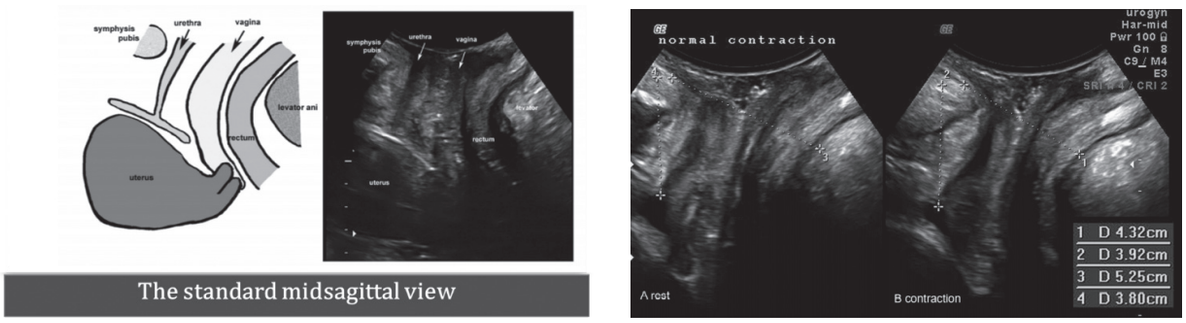


Picture 1 The midsagittal view measure the difference between the anteroposterior hiatal dimension in the resting stage compared to maximum squeeze using 2D transperineal ultrasound.[17-19]



Picture A at rest, **Picture B** during muscle contraction. Difference point: 3-1 = 0.93

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The title page should include the following information: (1) the title of the manuscript; (2) the names of the author(s), including each author's highest academic degree or professional certification; (3) the departmental and institutional affiliation of each author, including city, state, and country; (4) the name, address, telephone number, fax number, and email address of the author responsible for correspondence, and (if different) the name and address to be used for reprint requests; (5) if relevant, a statement about any previous presentation of the data or findings in a preliminary report or abstract; (6) an abbreviated title of not more than 45 characters (including spaces), to be used as a running head in print and for search results online; and (7) a word count for the body of the text (ie, excluding the abstract and the references). Acknowledgement of financial support and potential conflicts of interest must be included and should be placed in the Acknowledgements section (see below).

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Original Articles should include a structured abstract of no more than 250 words. The following headings are suggested: Introduction/Objective, Design, Methods (or Interventions), Results, Conclusions, and Key words (3-5 words or phrases). If this list of headings is inappropriate, variations are permitted: for example, a study that involved no intervention would use the heading "Methods" rather than "Intervention"; or an analysis of an existing data set might use the heading "Methods" in place of both "Intervention". For brevity, parts of the abstract can be written in phrases rather than complete sentences (eg, "Design: Retrospective cohort study" or "Design: Before-after trial").

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The main sections and subdivisions of the body text should be indicated by side heads flush with the left margin and two lines above the text. Keep Methods, Results, and Discussion distinct and separate. The Methods section should provide detail sufficient to allow others to re-create your experiment. Methods may not be described or restated in figure legends or table notes, but must be all together in the Methods section. The Results section contains the previously unpublished data derived by this application of your methods, without commentary (beyond the minimum that might be necessary to ensure intelligibility to the reader). The Discussion section contains your interpretation of the reported data and comments on its meaning. There should be no separate section labeled "Conclusion." Avoid duplicating in the text data that have been provided in tables or figures (minimal duplication, for emphasis or clarity, is acceptable). Also avoid duplication within the text; for example, the Discussion section should not restate all the findings that have been presented in Results and/or in tables and figures.

Acknowledgments

Financial support. Information on financial support should be provided by authors. The Acknowledgments section should list all sources of financial support for the work, including any financial arrangement with a company whose product is related to the study. If there was no financial support, that too should be stated. Acknowledgments, including grant support, should be placed at the end of the text.

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Journal article in press (example)

3. Figueroa P, Johanssen KL, Price FG, et al. Outbreak of Acinetobacter infection in a neonatal intensive care unit. J (in press).

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Book (example)

5. Heoprich PD. Infectious Diseases. *2nd ed*. New York, NY: Harper & Row; 1977.

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6. Schaffner W. Psittacosis: ornithosis, parrot fever. In: Beeson PB, McDermott W, Wyngaarden JB, eds. *Cecil Textbook of Medicine. 15th ed*. Philadelphia, PA: W. B. Saunders; 1979:336-338.

Web page (example)

7. Clinical laboratory fee schedule. Centers for Medicare and Medicaid Services website. https://www.cms.gov/ClinicalLabFeeSched/02_clinlab.asp#TopOfPage. Published 2010. Accessed April 2, 2010.

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Every column in a table requires a head that describes the contents of the cells below. The units of measure for all data must be clearly stated in the heads, in the stub (leftmost) column, or in data cells, as appropriate. Do not use vertical lines, and do not use ditto marks for repeated information.

List and define any abbreviations in a note below the table, above the table footnotes (no footnote designator is required for this line), even if the abbreviations have been defined in the text. Use superscript letters for footnote designators.

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Example of Tables:

Table 1 Characteristics of study subjects

Characteristics	Congenital hypothyroidism		Non Congenital hypothyroidism		*P*-value
	N	%	N	%	
Gender					
Male	40	64.2	171	53.4	.108
Female	22	35.5	149	46.6	
Birth weight (g)					
< 2,500	13	21.0	56	17.8	560
≥ 2,500	49	79.0	258	82.2	
Gestational age (Week)					
< 36	6	15.8	32	11.0	.415*
≥ 36	32	84.2	260	89.0	
APGAR at 5 minutes					
Normal (> 6)	59	100	302	97.4	.365*
Abnormal (≤ 6)	0	0	8	2.6	
Maternal age (Year)					
< 20	1	1.9	46	45.7	.024
20 - 35	45	83.3	208	71.0	
> 35	8	14.8	39	13.3	
Maternal history of thyroid hormone disease					
Yes	12	19.7	2	0.6	< .001
No	49	80.3	309	99.4	
Sepsis					
Yes	6	10.2	42	13.5	.485
No	53	89.8	269	86.5	
Ototoxic					
Yes	11	18.64	103	33.0	.028
No	48	81.4	209	67.0	
TSH screening(mU/L) Mean (Standard deviation)	29.5 (46.6)		5.4 (3.8)		< .001*
TSH at diagnosis(mU/L) Mean (Standard deviation)	20.4 (27.1)				
FT4 at diagnosis(ng/dL) Mean (Standard deviation)	1.4 (0.8)				

Abbreviations: TSH, Thyroid-stimulating hormone; FT4, Free thyroxine; APGAR, Activity Pulse Grimace Appearance and Respiration

Table 3 Univariable risk regression of the hearing loss

Characteristics	Risk ratio	95% CI	*P*-value
Congenital hypothyroidism	2.00	1.09-3.67	.0286
Ototoxic	2.37	1.34-4.19	.003
Sepsis	3.11	1.74-5.58	.0007

Abbreviations: CI, confidence interval

Figure

- Size: Image with a minimum of 35 x 14 cm. (w x h) using a minimum resolution of 300 dpi. Larger image, please use the same ratio.
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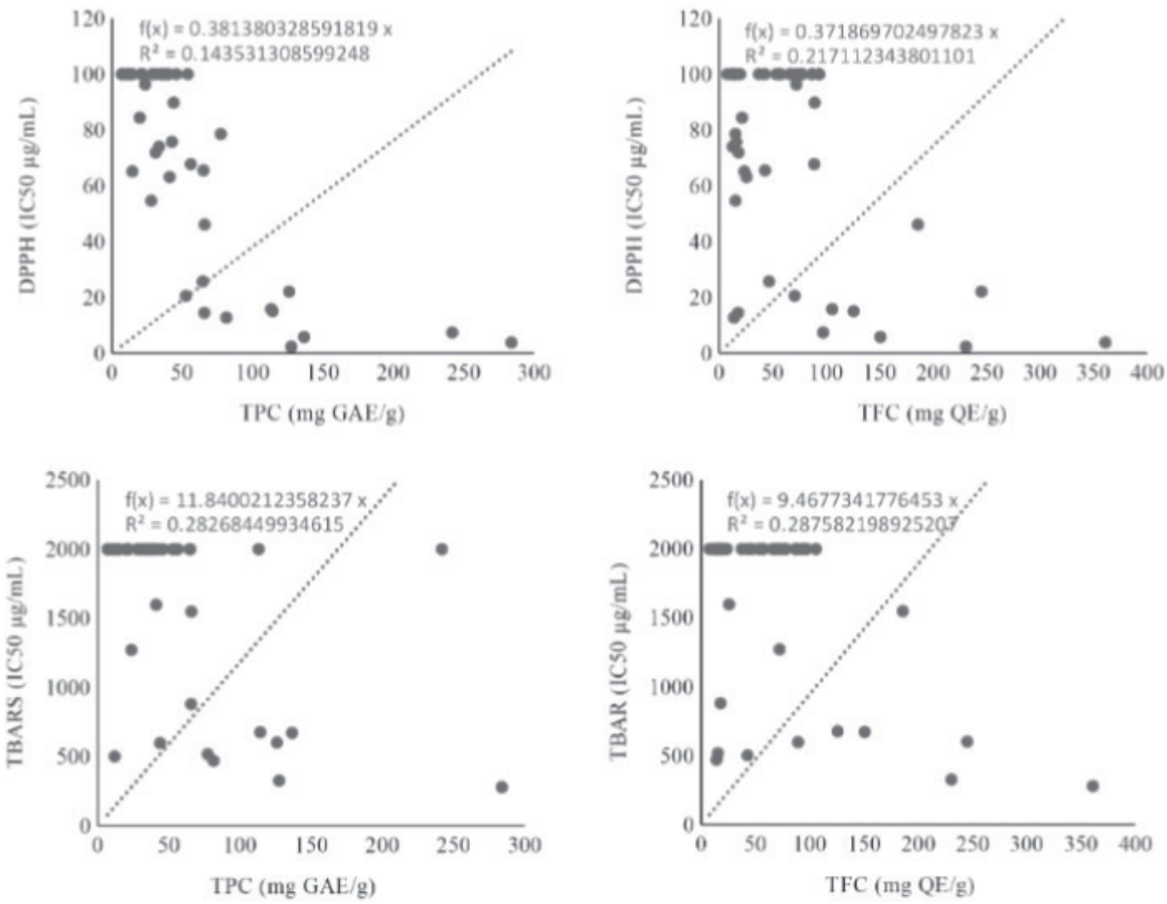

Figure 1 The Pearson statistical correlation scatters plot of the linear relationship between (A) DPPH and TPC, (B) DPPH and TFC, (C) TBARS and TPC, and (D) TBARS and TFC.

Example of Figure

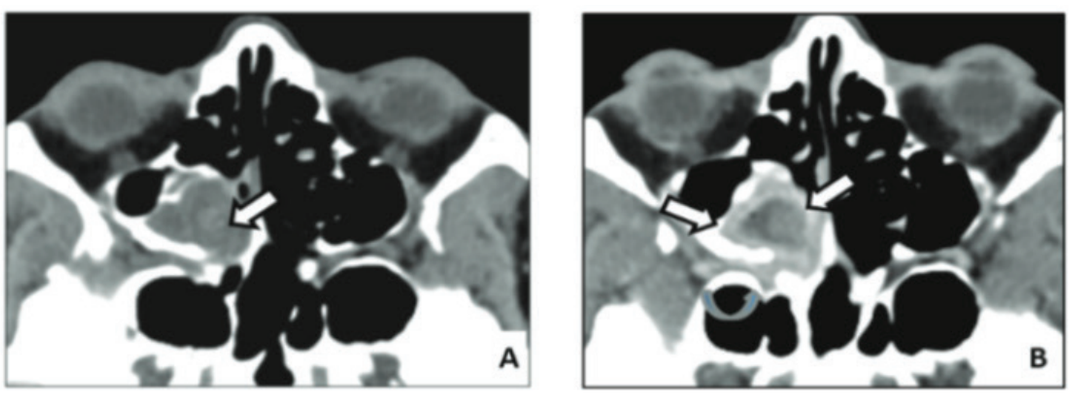

Figure 4 A and B: 58-year-old man with fungal ball at right ethmoid sinus was interpreted as inflammatory lesion. There is a hyperattenuating content (open arrow in A) with mucosal thickening at right posterior ethmoid sinus (open arrow in B) with fat haziness and increased enhancement at right PPF (curve arrow in B).