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Aims and scope

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1. Medical Technology
2. Physical Therapy
3. Occupational Therapy
4. Radiologic Technology
5. Communication Disorders
6. Other related fields

Submitted manuscripts within the scope of the journal will be processed strictly following the double-blinded peer review process of the journal. Therefore, the final decision can be completed in 1-3 months average, depending on the number of rounds of revision.

Objectives

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Types of manuscript

Manuscripts may be submitted in the form of review articles, original articles, short communications, as an approximate guide to length:

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- **Original articles** must not exceed 15 journal pages (not more than 3,500 words), including 6 tables/figures, and 40 reference (maximum 40, recent and relevant).
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High speed, low load training versus general exercise on quadriceps strength, physical performance and pain relief in individuals with knee osteoarthritis

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ABSTRACT

Background: Knee osteoarthritis (OA) is found generally among elderly. High speed, low load (HSLL) training is an alternative resistance exercise for elderly that improves muscle strength and physical function. Therefore, this home-based exercise using elastic bands may improve muscle strength and physical performance efficiently in elderly with knee OA.

Objectives: To determine the effects of HSLL training on quadriceps muscle strength, physical performance and pain in individuals with knee OA.

Materials and methods: Forty-one participants (6 males and 35 females) with a mean age of 65.05±7.15 years were divided randomly into HSLL group (n=20) and control (CON) group, who practiced general exercise (n=21). Both groups performed exercise 3 days/week for 8 weeks. For the outcome measures, the study considered the maximum voluntary isometric contractions torque (MVICT), 5 sit to stand test (5STST), 4-step stair climb test (4SSCT), 10m walk test (10MWT), knee injury and osteoarthritis outcome score-physical function short form (KOOS-PS), and visual analog scale (VAS) which were carried out before and after the training.

Results: MVICT increased significantly in HSLL and CON group after training (p<0.01 and p<0.05 respectively), whereas, the 10MWT, 4SSCT, KOOS-PS and VAS (p<0.01) decreased significantly. Although HSLL group provided a higher percentage of changes than CON group. However, no significant differences between the groups were evident for any measured parameters.

Conclusion: HSLL training and general exercise are beneficial for individuals with knee OA regarding the quadriceps muscle strength, physical performance and pain relief.

Introduction

Knee osteoarthritis (OA) is a most common condition that currently affects older adults. It is a major cause of disability among the elderly. Treatment to improve quality of life for older adults with knee OA has focused on improving measures of muscle strength, physical performance, and pain relief. The important principle of rehabilitation that improves symptoms of the disease is resistance training with a strengthening component.[1] Especially, quadriceps muscle that plays the key role for knee function and minimize knee pain among people with OA knee.[2] Therefore, quadriceps muscle strengthening exercise is crucial to improve performance of functional tasks. Normally for progression of the strength, the training program needs to use a high load with multiple repetitions but this type of program can induce pain, swelling and inflammation of the osteoarthritic knee due to a high

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compression force and over loading to the knee joint.[3,4] A study by Sayers and Gibson[5] suggested that training program with a high-speed but low load could improve a power of lower extremity as similar to the training program with a low-speed with high load. Current recommendations for resistance training in older adults with knee OA encourage strength-enhancing contractions at moderate to high resistances (60–80% of maximal strength or one repetition maximum [1RM]).[6] In recent years, high speed, low load (HSLL) training for older adults has been growing in the physical rehabilitation literature.[7-9] In high speed training, contractions are performed as fast as possible with lower resistance than traditional resistance training, and its goal is to improve the ability to produce force rapidly.[10] HSLL training has been considered potentially to produce more observable physical performance than those produced by traditional strength training.[11,12] In addition, it has been suggested that HSLL training has greater impact on explosive force production in older adults than high resistance training. A meta-analysis by Steib et al.[13] showed HSLL training was more effective than traditional slow speed resistance training for improving muscle power and physical performance in older adults.

To authors' knowledge, few pilot studies investigated the effect of HSLL training in older adults with knee OA. A study by Sayers et al.[14] also recruited a small number of participants (i.e. n=15 per group) and conducted the study in laboratory setting. The practice of high-speed training programs using an exercise machine in the laboratory might be difficult to apply in the community with the elderly having knee OA. A study by Pelletier et al.[15] conducted a case series study without control group, from which the results may be unclear in determining effectiveness beyond the control condition. Therefore, this study aimed to compare HSLL training, home-based exercise using elastic bands with general exercise that serve as a control group in evaluating the effects on quadriceps muscle strength, physical performance, knee function and pain in individuals with knee OA. This study hypothesized that HSLL training would have a greater impact on quadriceps muscle strength, physical performance and pain than general exercise training.

Materials and methods

Participants

Eligible participants with physician-diagnosed evidence of knee OA were aged 50 years or older and managed as outpatients at Tha Kham Health Center, Mae Hia district, Chiang Mai, Thailand had recruited for this study. The Inclusion criteria met the requirements of the American College of Rheumatology (ACR) clinical classification tree of knee OA [16], which consisted of knee pain including 3 paradigms. 1) the presence of knee pain without crepitus on motion, but bony enlargement on examination, 2) crepitus on active motion, morning stiffness of more than 30 minutes duration and bony enlargement, and 3) crepitus on active motion, morning stiffness of less than or equal to 30 minutes duration and age of over or equal to 38 years. The exclusion criteria consisted of history of heart disease, uncontrolled hypertension, total knee arthroplasty, lower extremity fracture in the previous 6 months, inability to give informed consent and current participation in structured exercise. Participants were randomized by using a stratified block randomization technique (4 participants per block) and were stratified by gender, age (50-59 years and ≥60 years) and pain severity (VAS 1-3 and VAS 4-6). Permuted block randomization in each block was then achieved using random numbers generated by Microsoft Excel for Windows. The protocol was approved by the ethics committee of the university and all of the participants provided written informed consent.

Procedures

This study was a single-blind 8 weeks intervention study to determine the effects of explosive HSLL training compared with the control (CON) group on quadriceps muscle strength, physical performance, knee function and pain. Outcome measures included maximum voluntary isometric contractions torque (MVICT) and physical performance measures which consisted of the 10m walk test (10MWT), 4-step stair climb test (4SSCT) and 5 sit to stand test (5STST). The physical performance measures are clinical (field) tests and also relevant to core activities commonly impaired in older adults with knee OA. The Knee Injury and Osteoarthritis Outcome Score-Physical Function-Short Form (KOOS-PS) also was used to assess self-reported knee function. In addition, the impact of knee pain on daily activity was evaluated on a 10-cm visual analogue scale (VAS). Participants were evaluated at baseline and 8-week after the exercise training program (about 30 minutes test period per time).

Training protocol

Participants exercised 3 times per week for 8 weeks as a home-based exercise training program. The duration of exercises was about 1 hour per time (exercise 6 minutes and rest 2 minutes per exercise). HSLL training was performed in seven exercises in 3 sets of 15 repetitions on both lower limbs: knee extension and flexion in sitting position without back support, hip abduction in standing position, sit to stand, step ups and downs and standing calf raises.[17] Knee extension, flexion and hip abduction exercises were performed using elastic bands (Thera-Band®) and their color indicated the resistance level (yellow, red, or green). Exercise loads (as indicated by band color) in resistance training were set at 30-45% of the maximal voluntary isometric force (MVIF)[18] at baseline assessment. The intensity of exercise was adjusted to 5% of the MVIF every 2 weeks. The color and length of the elastic bands used to perform the exercises were determined by the Page table.[19] In addition, participants in the HSLL training group were instructed to perform the concentric phase of each repetition as rapidly as possible and then return through the eccentric phase in 3 seconds. The (CON) group performed general exercises in 3 sets for 15 repetitions. The training combined with a range of motion exercises: knee flexion in supine and prone position and strengthening exercises: knee extension in supine position and supine position with hip flexion, knee flexion in prone position, knee extension in sitting position without back support and sit to stand.[20] The first 4 weeks of strengthening exercise had no resistance and the other side of the lower limb had taken as weight resistance thereafter. In addition, participants in the CON group performed both the concentric and eccentric phases

in 3 seconds.

For both groups, the researcher prescribed the exercise training program before starting program about 1 week. Moreover, the researcher personally visited participants at home every week. The monitoring compliance rate was deemed as 100%.

Measures posture

MVICT was tested by using a commercially available dynamometer (Con-Trex MJ), which allowed instantaneous isometric torque recording.[21] The Participants were seated comfortably on a dynamometer chair with their hip joint at about 85˚ (0˚= full extension). They were asked to position their arms across their chest with each hand clasping the opposite shoulder during the testing procedure. The knee angle was fixed at 60˚of flexion, which was the angle of maximal isometric force generation.[22] The participants were instructed to extend their leg as fast and forcefully as possible and sustained the contraction for 5 seconds. The test was conducted twice, separated by a 60 second rest interval and the highest peak torque was used for further analysis. Con-Trex software consistently indicated the duration of both contraction and rest phases.

Thus, the peak torque was determined as MVICT obtained after onset of the voluntary contraction. Isometric torque data were recorded from an isokinetic dynamometer with a sampling rate of 256 Hz and were exported and analyzed with Windows-based software. There was an excellent intra-rater reliability of the MVICT in the study considering the intra-class correlation (ICC) (3,1) =0.97.

The 10MWT[23] was evaluated over 10-m of floor length. The participants were provided with 2 m. in which to accelerate and decelerate before and after the test distance and asked to walk as quickly as possible. Two trials performed, separated by 5 minutes rest interval and the fastest time (s) was used for analyses. There was an acceptable intra-rater reliability of the 10MWT in the study considering the ICC (3,1) =0.87.

In the 4SSCT[24], the participants were instructed to ascend a flight of 4 stairs (16 cm. per step) as fast as they could, while being allowed to use a handrail if they thought it necessary for the purpose of safety. Two trials were performed, separated by 5 minutes rest interval and the fastest time (s) was used for analyses. There was an acceptable intra-rater reliability of the 4SSCT in the study considering the ICC (3,1) =0.86.

The participants in the 5STST[25] were seated in a hard-backed chair 43 cm. from the floor with their arms folded across their chest. They were instructed to rise to a full standing position as fast as possible and then return to the full-sitting position 5 times. Two trials were performed, separated by 5 minutes rest interval and the fastest time (s) was used for analyses. There was an acceptable intra-rater reliability of the 5STST in the study considering the ICC (3,1) value of 0.85.

Self-reported knee function was assessed by the KOOS-PS[26], with a 7-item Likert scale questionnaire scored from 0-4 (none, mild, moderate, severe, and extreme). In Possible raw score range of 0–28. Scores were then transformed to a range of 0-100, where 0 = no difficulty. There was an excellent intra-rater reliability of the KOOS-PS in the study considering the ICC (3,1) =0.94.

The severity of knee pain was evaluated by VAS. The Participants rated their pain level at the time of testing ("pain now"). The instrument was 10-cm horizontally, with anchor points of 0 (no pain) and 10 (maximum pain). There was an excellent intra-rater reliability of the VAS in the study considering the ICC (3,1) = 0.97.

Statistical analyses

This study found that the distribution of the data was not normal. Therefore, Wilcoxon Signed Rank tests were used to analyze differences between pre and post intervention. Mann-Whitney U-tests were performed to test the difference between the groups. In addition, the changes from baseline to post-intervention were compared between the groups in order to examine differences in the influences of intervention. The level of statistical significance was established at $p<0.05$. All statistical analyses were performed using the SPSS program (SN 5068035) for Windows.

Results

Demographics of the participants

Forty-one participants were randomized into 2 groups: HSLL (n=20), and CON (n=21) (Figure 1). Over the 8-week study period, 4 participants withdrew during the intervention, 1 in the HSLL group and 3 in the CON. Participants in HSLL group departed from the study due to lack of time. Among the respondents of control group who withdrew from the study, 2 of them had knee pain during the exercise and 1 had lack of time. Beside those, all of the other participants (HSLL n=19, CON n=18) completed the study.

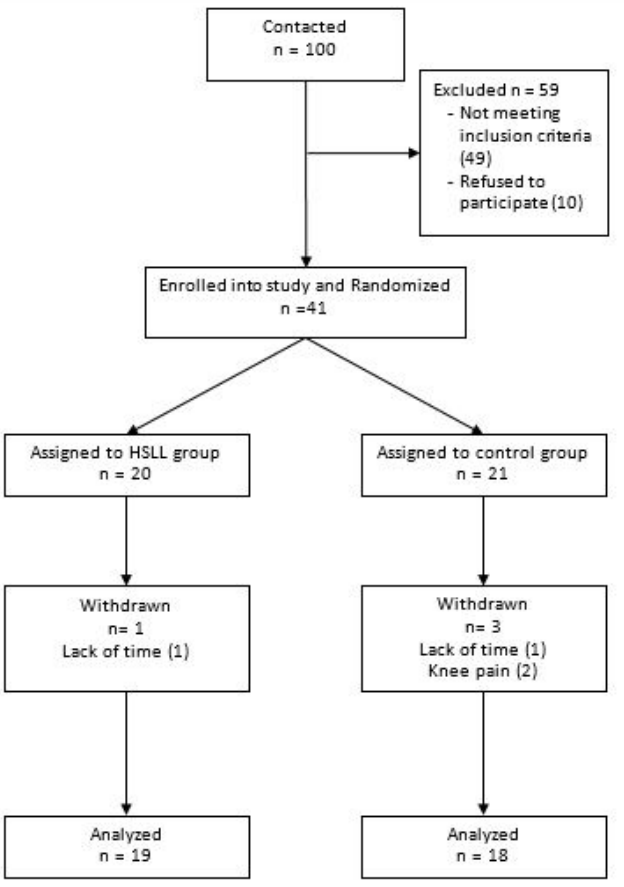

Figure 1 *Flowchart of study participation*

Baseline: There were no statistically significant differences among the groups with respect to age, height, weight, body mass index and duration of knee pain as shown in Table 1. There were no differences between the groups at baseline MVICT, 10MWT, 4SSCT, 5STST, KOOS-PS, or VAS (p>0.05) (Table 2).

Table 1 Demographic characteristic of participants (mean±SD).

Item	HSLL Group	Control Group	p value
Gender (female : male)	16:3	16:2	N/A
Age (y)	64.58±5.65	65.56±8.59	0.584
Height (cm)	152.55±10.11	152.75±8.70	0.605
Weight (kg)	60.61±11.89	61.57±10.58	0.796
BMI (kg/m2)	25.87±2.94	26.38±3.90	0.915
Duration of knee pain (y)	2.21±1.25	2.81±1.46	0.214

Note: HSLL: High Speed, Low Load Training, No significant differences between groups for demographic data (Mann-Whitney U test; p>0.05)

Baseline to post-training: There was significant increase in MVICT under the HSLL group that increased MVICT of 20.04% (p<0.01) compared to the CON group which increased MVICT about 17.87% (p<0.05). However, no significant differences in MVICT were observed between the groups (p>0.05). A significant decrease in the performance time for 10MWT was observed under the HSLL group, which decreased the 10MWT of 8.74% (p<0.05) compared to the CON group which decreased the 10MWT approximately 8.35% (p<0.05). However, no significant differences in 10MWT were observed between the groups (p>0.05). There was a significant decrease of the performance time for 4SSCT under the HSLL group which the 4SSCT was decreased approximately 34.60% (p<0.05) and CON group decreased about 26.41% (p<0.05). However, no significant differences in 4SSCT were observed between the groups (p>0.05). A significant decrease in KOOS-PS was observed under the HSLL group which the KOOS-PS was decreased about 31.1% (p<0.01) and CON group decreased about 32.57% (p<0.01). Nevertheless, no significant differences in KOOS-PS were observed between the groups (p>0.05). There was significant decreased in knee pain under the HSLL group which the VAS score decreased about 67.46% (p<0.01) and CON group decreased about 84.63% (p<0.01). Nonetheless, no significant differences in VAS were observed between the groups (p>0.05). No significant improvement in 5STST was observed in either groups (p>0.05) (Table 2).

Table 2 Changes in outcome measures (mean±SD) for the HSLL group and the control group.

Variable	HSLL Group (n=19)		Control Group (n=18)	
	Pre-intervention	Post-intervention	Pre-intervention	Post-intervention
MVICT	54.23±21.17	62.99±22.77*	59.86±17.56	67.26±13.68*
10MWT	4.36±0.86	3.95±0.71*	4.76±0.91	4.34±0.76*
4SSCT	3.04±0.96	1.85±0.48*	2.80±0.96	1.93±0.46*
5STST	10.51±2.09	10.41±2.04	11.26±2.84	11.01±1.56
KOOS-PS	36.58±8.30	24.69±11.26*	36.73±8.79	23.59±9.85*
VAS	4.47±1.26	1.32±1.46*	4.17±1.47	0.72±0.90*

*Note: MVICT: Maximal voluntary isometric contraction torque, 10MWT: 10m walk test, 4SSCT: 4 Steps stair climb test, 5STST: 5 Sit to stand test, KOOS-PS: Knee injury and osteoarthritis outcome score-physical function-short form, VAS: Visual analog scale, *p<0.05 for significant differences from pre-intervention to post-intervention, No significant differences between groups were demonstrated in all outcome measures.*

Discussion

The aim of this study was to evaluate the effects of HSLL training on quadriceps muscle strength, physical performance, knee function and pain in the elderly with knee OA for 8 weeks and compare with the CON group. The primary findings of this study suggested that exercise training improved in the MVICT, KOOS-PS and VAS and decreased in performance time for the 10MWT and 4SSCT in both groups. The 5STST did not change after the training period in either groups.

Muscle strengthening is known as an important element in most exercise programs for knee OA[1]. In this study, the both groups were increased in the MVICT, which followed the exercise training. HSLL training reportedly improved physical performance in older adults with knee OA[14] and those who were healthy.[8,9] The reduction of time in performing the 10MWT and 4SSCT in this study were also demonstrated in both groups. The quadriceps muscle and other lower extremity muscles might play an integral role in the stability, loading,

proprioception and functional movement of the knee joint[27], which possibly controls of tibial translation during ambulation and decreases shearing translation force. Furthermore, greater ability of the knee musculature to dissipate knee joint loads might decrease the risk of articular contact stress, which leads to pain.

Nevertheless, no significant differences were found between the groups in degrees of improvement in the 5STST, in which physical performance at fast movement was assessed. The participants in this study were older adults with knee OA, who had reduced leg muscle power because of degenerative change and pathology of symptoms. The loss of fast-twitch muscle fiber and falling from fast movement in the elderly might result from the inability of the leg muscle to produce vertical force and momentum.[28] In addition, a study by Turcot et al.[29] reported that adults with knee OA horizontally displace their center of mass by leaning their trunk forward and shift their weight on the contralateral side to unload the affected side by lateral trunk leaning. This mechanical strategy and compensation are proposed to reduce pain as well as decrease the action of quadriceps muscle (weaker muscles). Therefore, the biomechanics of chair raising in adults with knee OA not only have important factors of leg muscle strength and power, but also effective to adaptive movements that result in muscle activation. The participants in this study might not be aware of changing biomechanics or the effect of sit to stand exercise at home by themselves, and they may not perform quadriceps muscle activity as well as they should.

This study assessed knee function using the KOOS-PS and found that KOOS-PS in the HSLL and CON groups improved similarly. The extent of decreased in pain measured on the VAS was the same in following in the HSLL and CON groups. Previous studies of the elderly with knee OA reported improvements in knee function and pain due to resistance training.[15,30] Perhaps in the general exercise program, a combined range of motion and strengthening exercises was enough "intervention" to improve the measures of knee function and pain in this study.

The results of this study showed that the impact of HSLL training for older adults with knee OA increased in the percentage change of quadriceps muscle strength and physical performance significantly and subsequently, decreased KOOS-PS and pain less than general exercise training. The effect of resisted exercise training might facilitate muscle strength and induce some degrees of muscle fatigue and muscle soreness. Therefore, the result of the pain assessment after training in this study might be put together with these contributing factors (e.g. fatigue and soreness). Nevertheless, no significant differences were found between the groups in any dependent variables according to the protocol of this study. The results from this study would have significant implications for the further design of training programs for older people with knee OA, particularly to the HSLL resistance exercise training that occurring in more total volume per exercise session and longer periods of exercise programs (i.e. duration of exercise and training).[31] A study by Sayers et al.[14] recommended that training program in pathologic condition (i.e., degenerative osteoarthritis) especially in the aging population required more total volume per exercise session and longer periods of exercise programs (i.e., 12-14 repetitions/set, 3 sets/day, at least 3 days/week for at least 12 weeks) to demonstrated the significant changes in clinical outcomes.

This study had a few limitations. First, the number of participants was relatively small and the drop-out rate in the CON group was relatively high. The small sample size might pose the risk of type II errors. Second, the compliance rate relied on completion of a training diary by the participants, and exercise intensity was determined subjectively. These factors might interfere with the accuracy of the exercise prescription. Finally, this study compared the effects of exercise training program within 8-week period, and no long-term follow-up was traced.

Conclusion

The HSLL protocol in this study was not superior to the general exercise program. Both exercise programs (i.e., HSLL and CON) provide benefits for older adults with knee OA regarding quadriceps muscle strength, physical performance, knee function and pain relief. Future study should further explore the potential dose of the HSLL exercise program for knee osteoarthritis.

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Inhibitory effects of costunolide and parthenolide from Champi Sirindhorn (*Magnolia sirindhorniae*) on FLT3 protein expression in EoL-1 leukemic cells

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ABSTRACT

Background: FLT3 (Fms-like tyrosine kinase 3) belongs to the class III receptor tyrosine kinase that is involved in hematopoietic progenitor cell proliferation. It is a prognostic marker for acute myeloblastic leukemia (AML). To date, chemotherapy has been the most frequently used treatment for leukemia. It has had a very good outcome in the early stages of treatment. However, the main problem of chemotherapy is the side effects for leukemia patients, as it may also cause drug resistance after long time treatment. Magnolia (*Magnolia spp.*) is a medicinal plant and has been used as traditional medicine in China, Japan, and Thailand. It is used for treatment of gastrointestinal disorders, anxiety, allergic disease, etc.

Objectives: Effect of crude fractional extracts and purified active compounds from *Magnolia sirindhorniae* Noot. & Chalermglin (a new species of *Magnoliae spp*. which was discovered first in Thailand) were investigated for their cytotoxicity, leukemic cell proliferation, and FLT3 protein suppression in EoL-1 cells. Crude fractional extracts from leaves (fraction No. 1-3), twigs (fraction No. 4-6), and stems (fraction No. 7-9) were fractionated by hexane (fraction No. 1, 4, 7), ethyl acetate (fraction No. 2, 5, 8), methanol (fraction No. 3, 6, 9). The costunolide (1) and parthenolide (2) were purified from *n*-Hexane fraction from leaves and ethyl acetate fraction from twigs, respectively by column chromatography. Cytotoxicities against leukemic cells were determined by using MTT assay.

Results: Fraction No. 1, 2, 4, 5, 7, 8, costunolide (1), and parthenolide (2) showed strong cytotoxic effects on EoL-1 cells. Furthermore, the non-cytotoxic concentration (20% inhibitory concentration (IC_{20}) values) also decreased FLT3 protein expressions and total cell numbers of EoL-1 cells after treatments. Interestingly, fraction No. 1, 5, costunolide (1), parthenolide (2) decreased the FLT3 protein levels in a time- and dose-dependent manner.

Conclusion: In summary, costunolide and parthenolide are effective compounds from leaves and twigs of *M. sirindhorniae* to suppress FLT3 protein expression and cell proliferation.

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Introduction

Magnolia sirindhorniae Noot. & Chalermglin is a Thai medicinal plant that belongs to the family Magnoliaceae. It was found in Thailand in 1999 by Dr. Piya Chalermglin. Thai common name is "Champi Sirindhorn" (Nooteboom and Chalermglin, 2000). In Thailand it is named after HRH Princess Sirindhorn. Among Magnolia species, *M. obovata* and *M. officinalis* are very important in traditional Chinese and Japanese herbal medicines. Magnolia bark and flower have been used for treatment of gastrointestinal disorder, anxiety, and allergic disease. In addition, the bark showed anti-cancer[1], anti-inflammatory[2], and anti-oxidant activities.[3] In the central nervous system, it showed anti-stress, anti-anxiety[4], anti-depressant[5], anti-Alzheimer, and anti-stroke effects.[4] In cardiovascular system, it showed anti-esophageal obstruction, anti-gastric ulcer, anti-diarrhea, and hepatoprotective effects.[6] Moreover, magnosalin, a compound isolated from "shin-i" (Flos magnoliae), showed anti-arthritic[7], anti-angiogenetic[8], and anti-inflammatory activities.[9] The leaves of *M. sirindhorniae* collected from Khlong Luang District, Pathum Thani Province, Thailand have been reported to contain five sesquiterpene lactones including costunolide, santamarine, reynosin, parthenolide, and lipiferolide.[10]

Sesquiterpene lactone compounds had cytotoxicity on U937[1], bladder cancer cells[11], multiple myeloma[12], M DA-MB231,[13] HL-60, and L1210 cell lines.[14] Costunolide from stem bark of *M. sieboldii* has been reported to induce apoptotic cell death in a dose-dependent manner and decrease Bcl-2 protein (anti-apoptotic protein), whereas the cleavage poly-(ADP-ribose) polymerase was activated in Colon 26, 3LL Lewis, J82, T24, and HL-60 cell lines.[15] Costunolide (10 μM) from stem bark of *M. sieboldii* also demonstrated to trigger apoptosis in U937 cells by depleting intracellular reduced glutathione (GSH) and protein thiols.[1] In addition, it could inhibit growth and telomerase activity of human breast carcinoma cells (MCF-7 and MDA-MB-231) in a dose- and time-dependent manner.[16] Moreover, costunolide, isolated from roots of *Saussurea loppa* (Mu Xiang), has been reported to induce apoptosis in bladder cancer cells by mediating through ROS generation and mitochondrial dysfunction.[11] However, the activity of crude fractional extracts and purified active compounds from Thai *M. sirindhorniae* on leukemic cells have never been reported, especially its effect on molecular target protein involved in leukemic cell proliferation. Feline McDonough Sarcoma (FMS)-like tyrosine kinase 3 or FLT3 protein and its mutations in leukemic cells are also involved in leukemic cell proliferation. It primarily expressed on committed myeloid and B-lymphoid progenitors and plays an important role in their survival, proliferation, and differentiation.[17] Low levels of FLT3 protein expression have been found in normal peripheral blood mononuclear cells (PBMCs). In contrast, overexpression of FLT3 protein has been found in leukemic blood cells, especially in acute myeloid leukemia (AML) and B-cell acute lymphoblastic leukemia (B cell ALL).[18] Parthenolide, an active compound from feverfew plant (*Tanacetum parthemium*) has been investigated for the anticancer activity and reported the effect to induce apoptosis in pre-B acute lymphoblastic leukemia cell lines, including cells carrying chromosomal translocation.[19] In addition, it could induce apoptosis through mitochondrial cytochrome C release, and caspase activation in chronic lymphocytic leukemia (CLL).[20] It stimulated the modification of redox state of critical exofacial thiols in Granta mantle lymphoma cells.[21] Furthermore, it has been reported the induction of autophagy through ROS generation, GSH depletion, JNK activation, and inhibition of NF-kB activity in human breast cancer cell line, MDA-MB231.[13]

However, the effect of crude fractional extracts and purified costunolide and parthenolide from *M. sirindhorniae* on FLT3 protein expression in leukemic cells is still unknown. The present study was designed to investigate the cytotoxicity and inhibitory effects of crude fractional extracts (hexane, ethyl acetate, and methanol) and purified active compounds (costunolide and parthenolide) on FLT3 protein expression. EoL-1 cell line was used as a leukemic cell model for this study because it shows high level of endogenous FLT3 protein expression.

Materials and methods

Chemical materials

RPMI 1640 (Invitrogen™, CA, USA), fetal bovine serum (FBS), *L*-glutamine, penicillin/streptomycin were purchased from Gibco (Invitrogen™, CA, USA), MTT dye, commercial costunolide (costunolide (3)) and commercial parthenolide (parthenolide (4)) were purchased from Sigma-Aldrich (St Louis, MO, USA). Trypan blue dye solution was purchased from AMRESCO® (Solon, OH, USA). Rabbit polyclonal anti-FLT3 antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Rabbit polyclonal anti-GAPDH (Glyceraldehyde phosphate dehydrogenase) antibody was purchased from Santa Cruz Biotechnology (CA, USA). HRP conjugated goat anti-rabbit IgG was purchased from Invitrogen™ Life (Carlsbad, CA, USA). Enhanced chemiluminescence detection kit was purchased from Thermo Scientific (Miami, USA). Luminata™ Forte Western HRP Substrate was purchased from Millipore Corporation (Billerica, MA, USA). *n*-Hexane, ethyl acetate, and methanol were purchased from Merck (Darmstadt, Germany).

Plant materials

Leaves, twigs, and stems of *M. sirindhorniae* were collected from the Thailand Institute of Scientific and Technological Research, Khlong Luang District, Pathum Thani Province, Thailand, in April 2010. A voucher specimen No. BKF420621 was deposited at the herbarium of the Royal Forest Department, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. Herbarium specimen has been studied and annotated by traditional methods of herbarium taxonomy.

Extraction and isolation of M. sirindhorniae

Total nine crude fractional extracts (fraction No. 1-9), fraction No. 1-3 were extracted from leaves of *M. sirindhorniae* by *n*-hexane (Hex), ethyl acetate (EtOAc), and methanol (MeOH) respectively, fraction No. 4-6 were extracted from twigs by Hex, EtOAc, and MeOH, respectively. Fractions No. 7-9 were extracted from stems by Hex, EtOAc, and MeOH, respectively. In addition, the active fractions were investigated by separation on column and preparative thin layer chromatographic (TLC) methods until purified 1 and 2 were obtained. The

two compounds (1 and 2) were identified as costunolide (1) and parthenolide (2).[10] To identify the compounds, spectroscopic analyses, including infrared spectroscopy, electrospray ionization-mass spectrometry, and ^{1}H and ^{13}C nuclear magnetic resonance (NMR), were performed. Two-dimensional-NMR measurements, such as correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond correlation (HMBC), also supported the identifications. Structures of costunolide (1) and parthenolide (2) are shown in Figure 1A and 1B, respectively. Costunolide (1) and parthenolide (2) were further investigated for their effects on cytotoxicity and FLT3 protein expression.

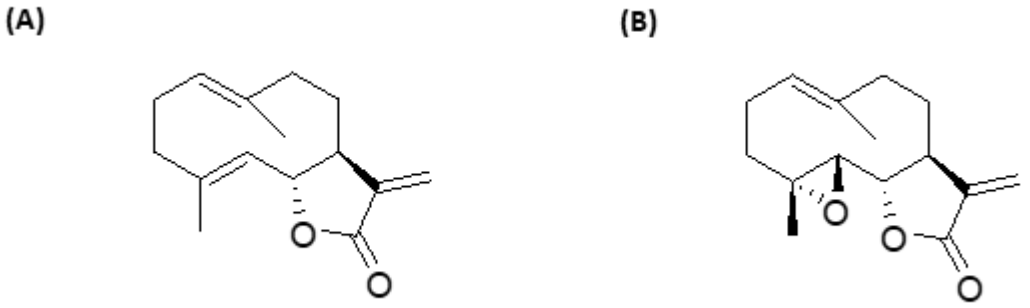

***Figure 1**. Chemical structure of (A) costunolide (1) and (B) parthenolide (2)*

Cells and cell culture conditions

EoL-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1 mM *L*-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin, and incubated under 80% relative humidity with 5% CO_2 at 37 °C.

MTT cytotoxicity assay

Cytotoxicity of crude fractional extracts (fraction No. 1-9), costunolide (1) and parthenolide (2) were evaluated using the MTT assay. Briefly, EoL-1 cells (5.0×10^4 cells/well) were cultured in 96 well plates containing 100 µL medium prior to treat for 24 hrs. After that, 100 µL of fresh medium containing various concentrations (0-100 µg/mL) of the test compounds were added to each well and incubated for 48 hrs. MTT dye solution was added (15 µL/100 µL medium) and the plates were incubated at 37 °C for 4 hrs in a humidified 5% CO_2 atmosphere. Afterward, 200 µL of DMSO were added to each well, and mixed thoroughly to dissolve the dye crystals. The absorbance was measured using an ELISA plate reader (Biotek EL 311) at 570 nm with a reference wavelength of 630 nm. High optical density readings corresponded to a high intensity of dye color represent to a high number of viable cells able to metabolize MTT salts. Fractional absorbance was calculated by the following formula:

$$\% \text{ Cell viability} = \frac{\text{Mean absorbance in test well}}{\text{Mean absorbance in vehicle control well}} \times 100$$

Average cell survival obtained from triplicate determinations at each concentration was plotted as a dose response curve. The experiment was done in 3 independent experiments. The 50% inhibitory concentration (IC_{50}) of the active substances was determined as the lowest concentration which reduced cell growth by 50% in treated compared to untreated culture or vehicle control culture (0.2% DMSO in culture medium). IC_{50} values were mean±standard deviation (SD) and compared for their activities.

Trypan blue exclusion test

Cell viability was measured by trypan blue dye exclusion method. Cells were treated with various concentrations of crude fractional extracts (fraction No. 1-9), purified active compounds (costunolide (1) and parthenolide (2)), and commercial compounds (costunolide (3) and parthenolide (4)). Then, cells and 0.4% trypan blue dye were mixed and counted using a light microscope. All experiments were performed in triplicate.

Protein extraction and Western blotting

EoL-1 cells were treated with crude fractional extracts (fraction No. 1-9), purified active compounds (costunolide (1) and parthenolide (2)), and commercial compounds (costunolide (3) and parthenolide (4)) for 48 hrs, after cell harvesting, cells were washed twice with cold PBS, pH 7.4, and lysed with cold RIPA buffer (50 mM Tris, 0.1% SDS, 1% Triton X-100, 150 mM NaCl, 0.5 mM EDTA, and 0.001% protease inhibitor cocktail) for whole protein extraction. Whole protein lysate (100 µg) were loaded onto 12% SDS-PAGE and then transferred to PVDF membranes (Merck and Millipore, Burlington, MA, USA). Membranes were blocked with 5% skim milk and probed with rabbit anti-FLT3 at 1:1,000 dilution. Rabbit anti-GAPDH at a dilution of 1:1,000 was used for protein loading control. The reaction was followed by HRP-conjugated anti-rabbit IgG at 1:20,000 dilution. Proteins were visualized using an enhanced chemiluminescence detection kit (Luminata™ Forte Western HRP Substrate). Densitometry was performed using Alpha Innotech software. Band density of the loading control was used to normalize the band densities of proteins of interest to obtain the relative normalized expression level as compared to the exposed control.

Statistical analysis

All data were expressed as mean±SD from triplicate samples of three independent experiments. Statistical differences between the means determined using One-way

analysis of variance (One-way ANOVA). The differences were considered significant when the probability value obtained was found to be less than 0.05 (p<0.05) and 0.01 (p<0.01).

Results and discussion

M. sirindhorniae extracts

All *M. sirindhorniae* extracts were separately evaporated to dryness under reduced pressure at 40 °C to give 9 crude fractional extracts. Their extracts were brownish sticky solid. Crude MeOH extract of leaves showed the highest yield (6.23%), followed by crude MeOH extract of stem (5.39%), and crude EtOAC extract of leaves (4.89%), respectively (Table 1).

Active fractions were repeated column chromatography of the Hex extract of leaves resulted in the isolation of costunolide (1) and parthenolide (2), while two compounds were major constituent in these crude fractions.

Percent of costunolide contents in fraction No. 1, 2, 3, 5, 7, and costunolide (1) was identified by HPLC and compared their retention time with the internal standard costunolide (3). The results showed that percent of costunolide contents were 7.01, 1.09, 0, 0, 0, and 49.6%, respectively. In addition, parthenolide's peaks in the samples (fraction No. 5, 7, and parthenolide (2)) were also identified by HPLC and compared their retention time to the internal standard parthenolide (4). The results showed that parthenolide contents were 39.60, 20.87, and 78.36%, respectively.

Table 1 Percentage of crude fractional extracts No. 1-9.

Crude fraction No.	Plant part	Solvent for extraction	Weight (gm)		% yield
			Dry plants	Extracts	
1	Leave	*n*-Hexane	310.2	9.42	3.03
2	Leave	Ethyl acetate		15.19	4.89
3	Leave	Methanol		19.31	6.23
4	Twig	*n*-Hexane	309.5	6.53	2.11
5	Twig	Ethyl acetate		8.07	2.61
6	Twig	Methanol		12.12	3.92
7	Stem	*n*-Hexane		8.61	2.76
8	Stem	Ethyl acetate	312.3	11.76	3.76
9	Stem	Methanol		16.84	5.39

Cytotoxicity of crude fractional extracts and purified compound in EoL-1 cell line

Cytotoxic effects of crude fractional extracts (No. 1-9), costunolide (1), and parthenolide (2) from Champi Sirindhorn on EoL-1 cells have been shown in Figure 2. Crude fraction No. 1, 2, 4, 5, 7, 8, costunolide (1), parthenolide (2), costunolide (3), and parthenolide (4) showed cytotoxicity on EoL-1 leukemic cells at 48 hrs with the IC_{50} values of 35.6, 50, 32.0, 39.6, 24.0, 46.9, 5.5, 3.9, 1.1, 2.1 µg/mL, respectively. On the other hand, crude fraction No. 3, 6, and 9 had no cytotoxic effects (IC_{50}>100 µg/mL).

Effects of fractional extracts and purified compounds on FLT3 protein expression in EoL-1 cell line

IC_{20} (obtained from MTT assay) values of crude fractional extracts and purified compounds were used to examine their effects on FLT3 protein expression and compared to commercial compounds (costunolide (3) and parthenolide (4)). FLT3 protein levels were decreased after treatments with 20, 36, 100, 19, 29, 100, 10, 31, and 100 µg/mL of fraction No. 1-9 in EoL-1 cells by 43.5, 22.0, 5.7, 38.2, 62.1, 9.0, 33.8, 4.9, and 0%, respectively when compared to the vehicle control at 48 hrs (Figure 3A). These treatments also significantly decreased the total cell numbers at 48 hrs by 77.4, 62.5, 29.7, 78.4, 84.5, 28.0, 62.9, 26.1, and 15.8%, respectively (Figure 3B). Non-cytotoxic doses of costunolide (1) (3.9 µg/mL), parthenolide (2) (1.0 µg/mL), costunolide (3) (0.9 µg/mL), and parthenolide (4) (0.8 µg/mL) were examined and compared to those of fractions. Costunolide (1), parthenolide (2), costunolide (3), and parthenolide (4) decreased FLT3 protein expression by 66.0, 78.3, 91.5, and 90.4%, respectively when compared to the vehicle control (Figure 3C). Furthermore, costunolide (1), parthenolide (2), costunolide (3), and parthenolide (4) significantly decreased the total cell numbers at 48 hrs by 81.9, 84.3, 91.0, and 88.3%, respectively when compared to vehicle control (Figure 3D).

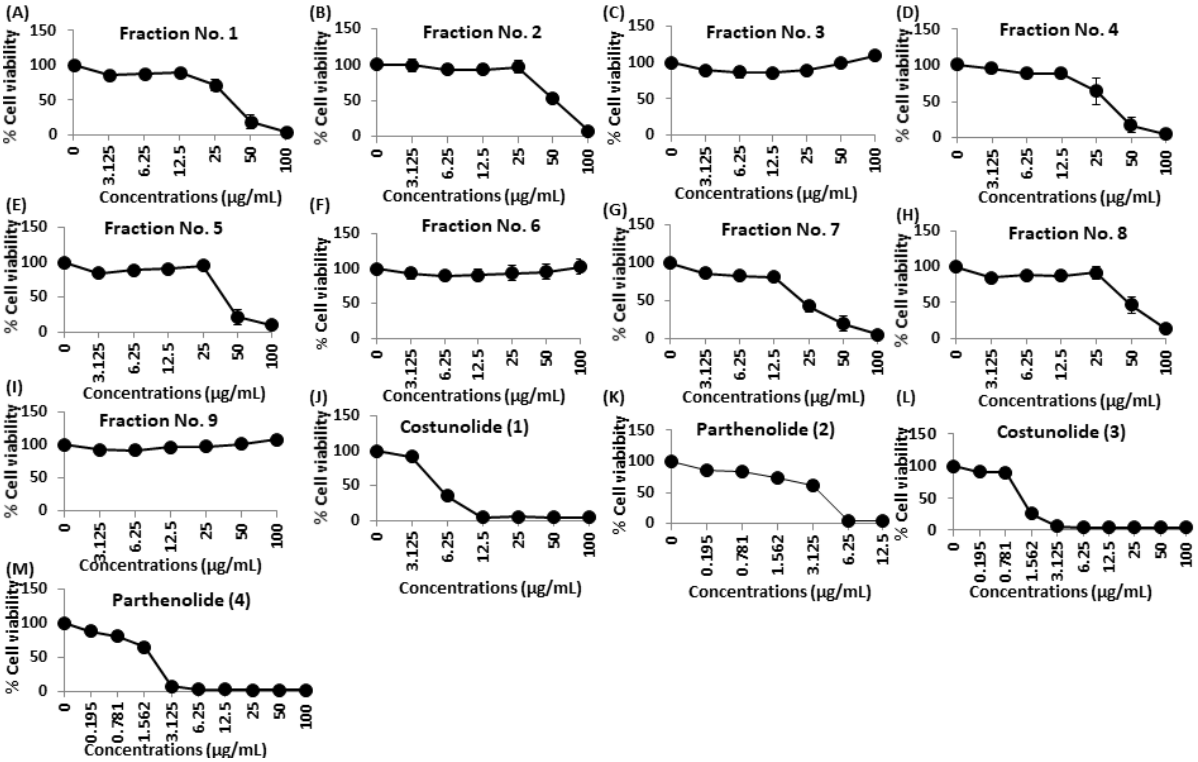

Figure 2. *cytotoxic effects of crude fractional extracts (No. 1-9) (A-I), costunolide (1), parthenolide (2) from Champi Sirindhorn (J-K), costunolide (3), and parthenolide (4) from Sigma-Aldrich (L-M) against EoL-1 cell line. Average of cell viability was obtained from three independent experiments.*

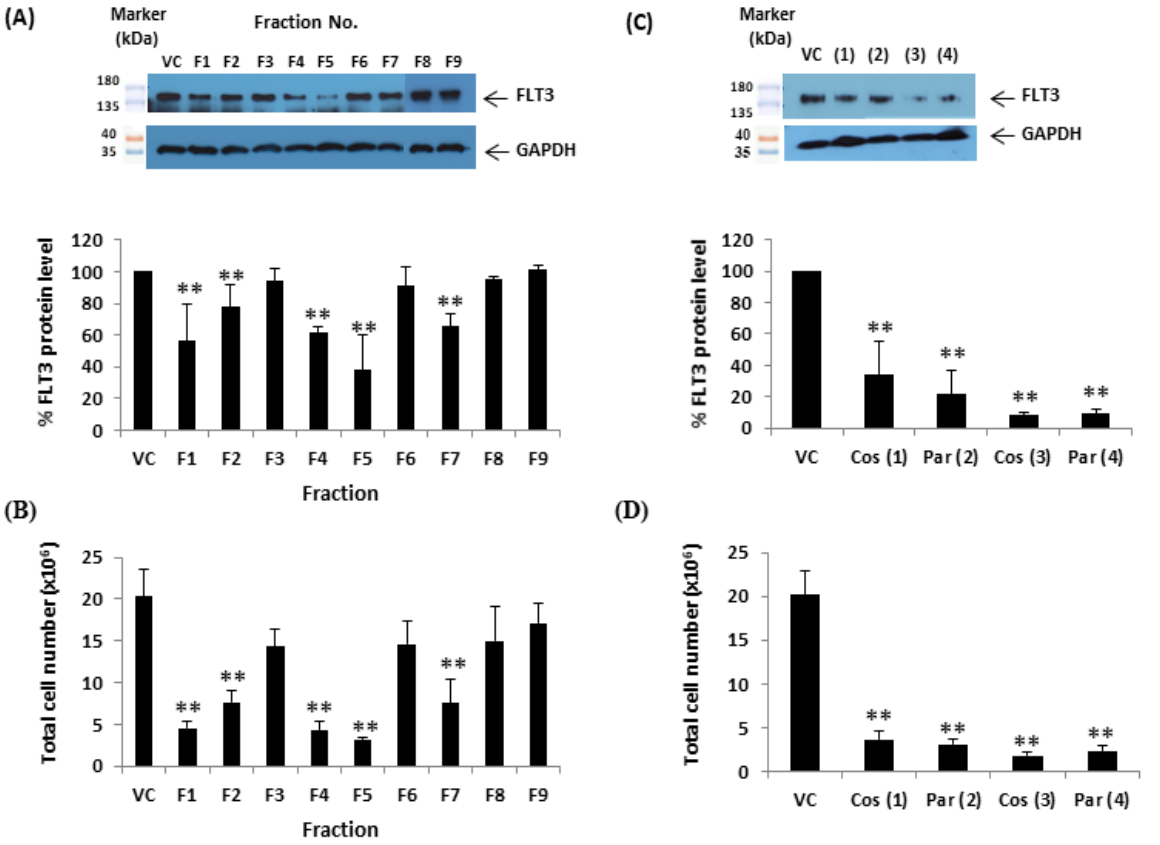

Figure 3. *Effect of fraction No.1-9 (F1-9), costunolide (1), parthenolide (2), costunolide (3), and parthenolide (4) on FLT3 protein expression in EoL-1 cells by Western blotting. (A and B) Levels of FLT3 protein expression and total cell numbers after treatment with 20, 36, 100, 19, 29, 100, 10, 31, and 100 µg/mL of F1-9, respectively for 48 hrs. (C and D) Levels of FLT3 protein expression and total cell numbers after treatment with 3.9, 1.0, 0.9, and 0.8 µg/mL of costunolide (1), parthenolide (2), costunolide (3), and parthenolide (4), respectively for 48 hrs. GAPDH was used as a loading control. (VC: vehicle control, F1-9: fraction No.1-9, Cos (1): purified costunolide (1), Par (2): purified parthenolide (2), Cos (3): commercial costunolide (3) and Par (4): commercial parthenolide (4)). Asterisk (**) denotes a significant difference from control group ($p<0.05$ and $p<0.01$).*

Effect of time periods and doses of fraction No. 1 (F1) on FLT3 protein expression and total cell numbers in EoL-1 cell line

Treatment of EoL-1 cells with F1 (high content of costunolide) for 6, 12, and 24 hrs decreased FLT3 protein expressions by 2.1, 0.9, and 49.2%, respectively, when compared to the vehicle control (Figure 4A). F1 significantly decreased FLT3 protein levels after treatments with various doses (10, 15, 20, and 25 µg/mL) at 24 hrs were decreased by 3.9, 22.0, 28.8, and 44.3%, respectively (Figure 4B). The total cell numbers at 6, 12, and 24 hrs were decreased by 16.9, 41.0, and 43.8%, respectively (Figure 4C). Total cell numbers in response to 10, 15, 20, and 25 µg/mL were significantly decreased at 24 hrs by 6.1, 21.4, 48.5, and 66.0%, respectively when compared to vehicle control (Figure 4D).

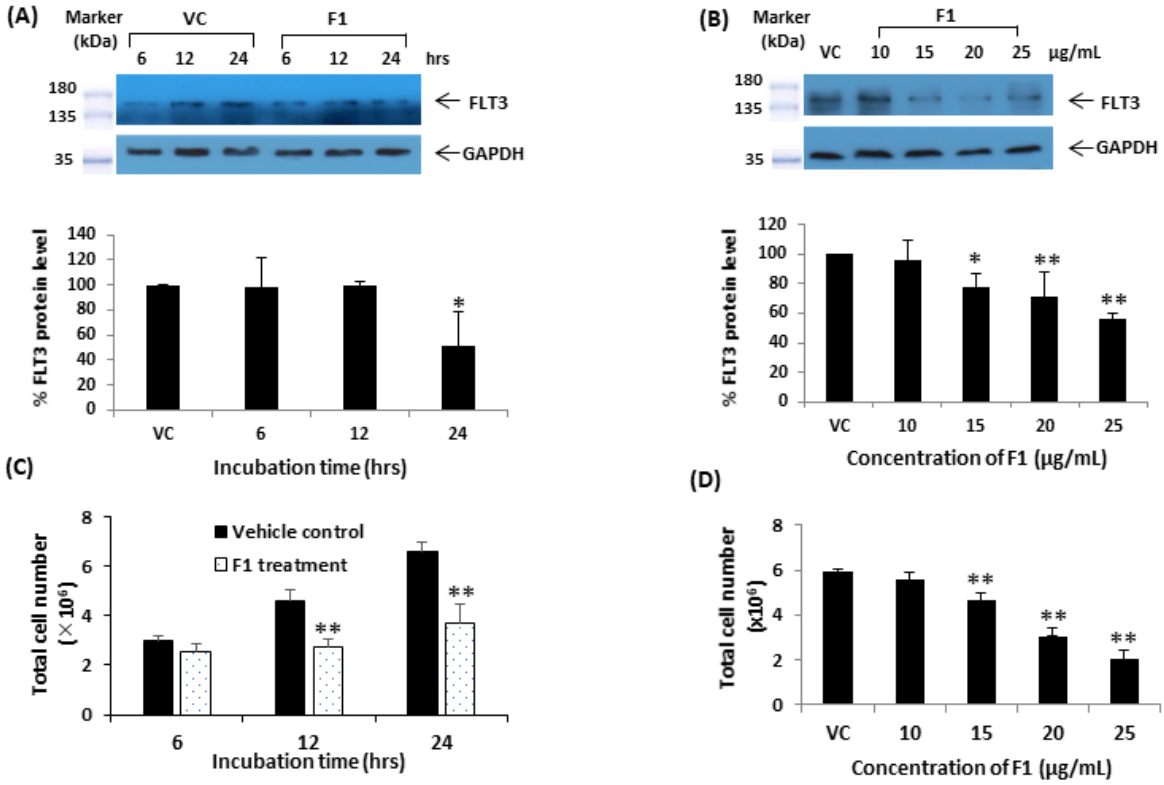

Figure 4. *Effect of various times and concentrations of fraction No.1 (F1) on FLT3 protein expression in EoL-1 cells by Western blot analysis. (A) EoL-1 cells were cultured with F1 at the concentration of 20 µg/mL for 6, 12, and 24 hrs. (B) EoL-1 cells were cultured with different concentrations of F1 (10, 15, 20, and 25 µg/mL) for 24 hrs. (A) FLT3 protein expression level was measured by Western blotting. GAPDH was used as loading control. (A and B) Densitometry was used to quantitate the protein levels and graph as the percentage of vehicle control (0.02% DMSO alone without the F1 in culture medium). C and D: Total cell numbers measured by trypan blue dye exclusion method. Data are mean±SD of three independent experiments. Asterisk (*) (**) denotes a significant difference from the control group ($p<0.05$ and $p<0.01$).*

Effect of time periods and doses of fraction No. 5 (F5) on FLT3 protein expression and total cell numbers in EoL-1 cell line

Treatment of EoL-1 cells with F5 (high content of parthenolide) for 3, 6, and 12 hrs decreased FLT3 protein expressions by 62.9, 69.8, and 89.8%, respectively, when compared to the vehicle control (Figure 5A). F5 significantly decreased FLT3 protein levels after treatments with various doses (20, 25, 30, and 35 µg/mL) at 12 hrs were decreased by 63.1, 72.9, 83.2, and 84.4%, respectively (Figure 5B). Total cell numbers at 3, 6, and 12 hrs were decreased by 20.0, 37.4, and 52.0%, respectively (Figure 5C). Total cell numbers in response to 20, 25, 30, and 35 µg/mL were significantly decreased at 12 hrs by 13.2, 21.0, 53.6, and 57.3%, respectively when compared to vehicle control (Figure 5D)

Effect of time periods and doses of purified costunolide (1) on FLT3 protein expression and total cell numbers in EoL-1 cell line

Treatment of EoL-1 cells with costunolide (1) for 3, 6, and 12 hrs decreased FLT3 protein levels by 70.8, 90.2, and 90.7%, respectively, when compared to the vehicle control (Figure 6A). Costunolide (1) significantly decreased FLT3 protein levels at 12 hrs by 32.4, 55.2, 73.4, and 77.7%, in response to 2, 3, 4, and 5 µg/mL, respectively (Figure 6B). The total cell numbers at 3, 6, and 12 hrs were decreased by 9.1, 28.1, and 50.7%, respectively (Figure 6C). Total cell numbers in response to 2, 3, 4, and 5 µg/mL were significantly decreased at 12 hrs by 25.2, 41.7, 58.5, and 71.5%, respectively when compared to the vehicle control (Figure 6D).

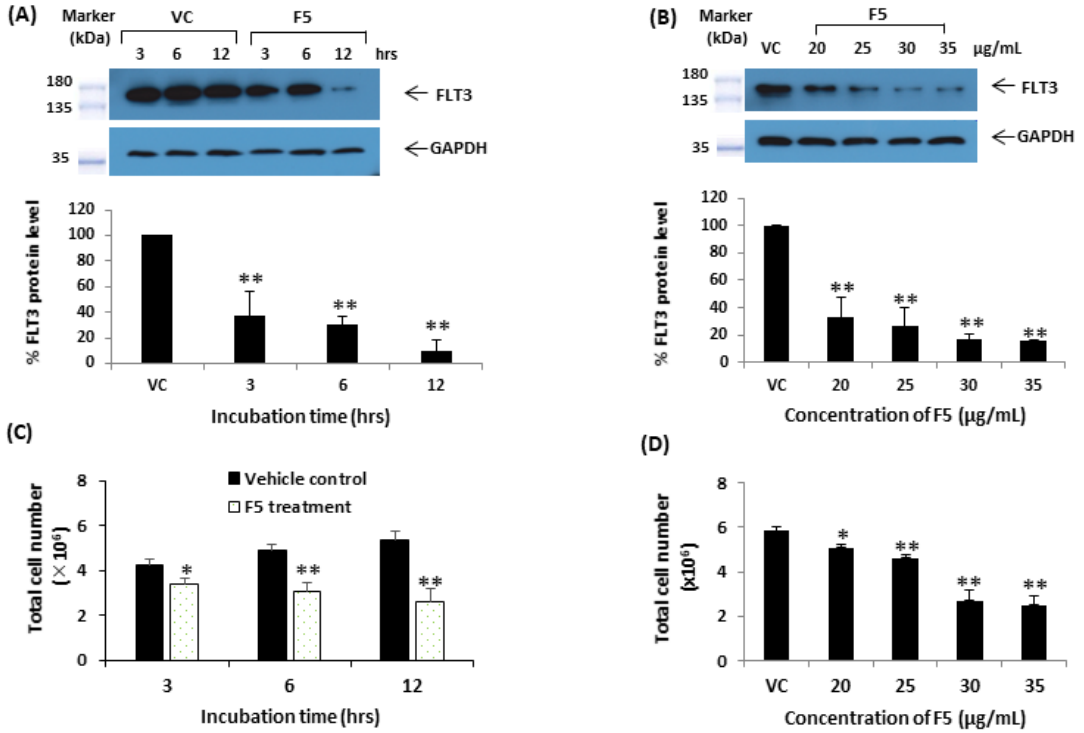

Figure 5. *Effect of various times and concentrations of fraction No.5 (F5) on FLT3 protein expression in EoL-1 cells by Western blot analysis. (A) EoL-1 cells were cultured with F5 at the concentration of 30 µg/mL for 3, 6, and 12 hrs. (B) EoL-1 cells were cultured with different concentrations of F5 (20, 25, 30, and 35 µg/mL) for 12 hrs. (A) FLT3 protein expression level was measured by Western blotting. GAPDH was used as loading control. (A and B) Densitometry was used to quantitate the protein levels and graph as the percentage of vehicle control (0.02% DMSO alone without the F5 in culture medium). C and D: Total cell numbers measured by trypan blue dye exclusion method. Data are mean±SD of three independent experiments. Asterisk (*) (**) denotes a significant difference from the control group (p<0.05 and p<0.01).*

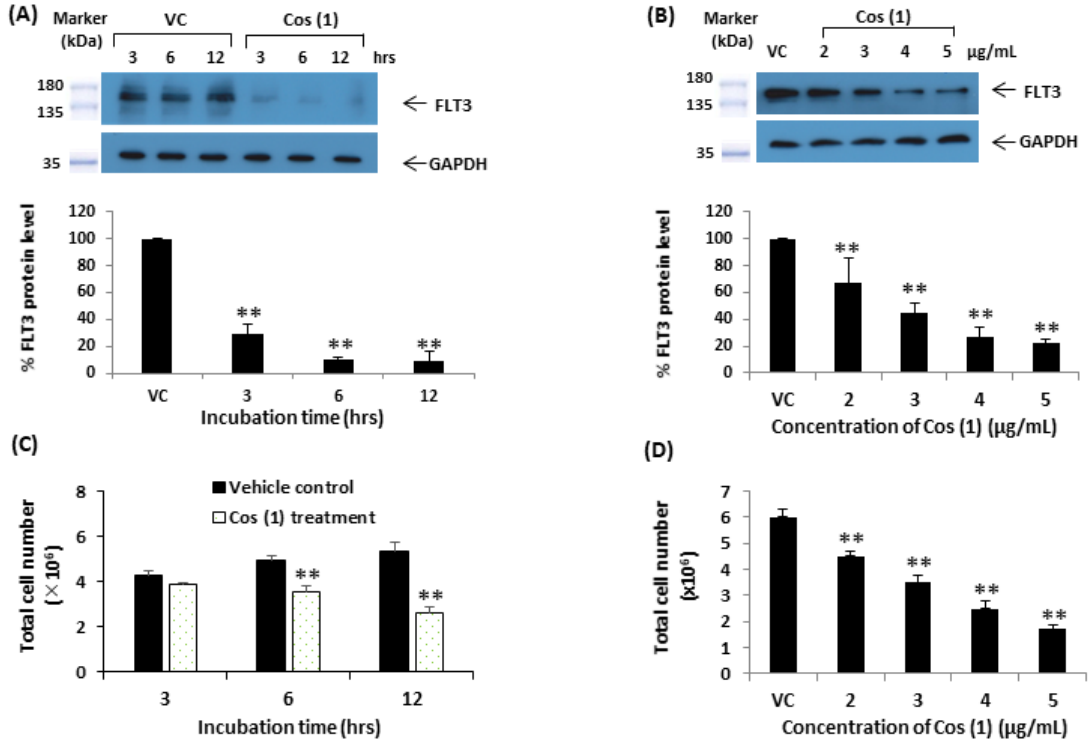

Figure 6. *Effect of various times and concentrations of costunolide (1) (Cos (1)) on FLT3 protein expression in EoL-1 cells by Western blot analysis. (A) EoL-1 cells were cultured with Cos (1) at the concentration of 4 µg/mL for 3, 6, and 12 hrs. (B) EoL-1 cells were cultured with different concentrations of Cos (1) (2, 3, 4, and 5 µg/mL) for 12 hrs. (A) FLT3 protein expression level was measured by Western blotting and GAPDH was used as loading control. (A and B) Densitometry was used to quantitate the protein levels as the percentage of vehicle control (0.02% DMSO alone without the Cos (1) in culture medium). C and D: Total cell numbers measured by trypan blue exclusion method. Data are mean±SD of three independent experiments. Asterisk (*) (**) denotes a significant difference from the control group (p<0.05 and p<0.01).*

ffect of time periods and doses of costunolide (3) on FLT3 protein expression and total cell numbers in EoL-1 cell line

In this study, costunolide (3) was used as a model to determine its effect on time periods and doses in EoL-1 cells. Treatment of EoL-1 cells with costunolide (3) for 1, 3, and 6 hrs decreased FLT3 protein levels by 22.2, 25.4, and 47.8%, respectively, when compared to the vehicle control (Figure 7A). Cos (3) significantly decreased FLT3 protein levels at 6 hrs by 52.8, 53.8, 64.3, and 85.7% in response to 0.5, 1.0, 1.5, and 2.0 µg/mL, respectively (Figure 7B). Total cell numbers at 1, 3, and 6 hrs were decreased by 4.6, 20.0, and 39.3%, respectively (Figure 7C). Total cell numbers in response to 0.5, 1.0, 1.5, and 2.0 µg/mL were significantly decreased at 6 hrs by 18.8, 37.7, 55.0, and 76.3%, respectively when compared to vehicle control (Figure 7D).

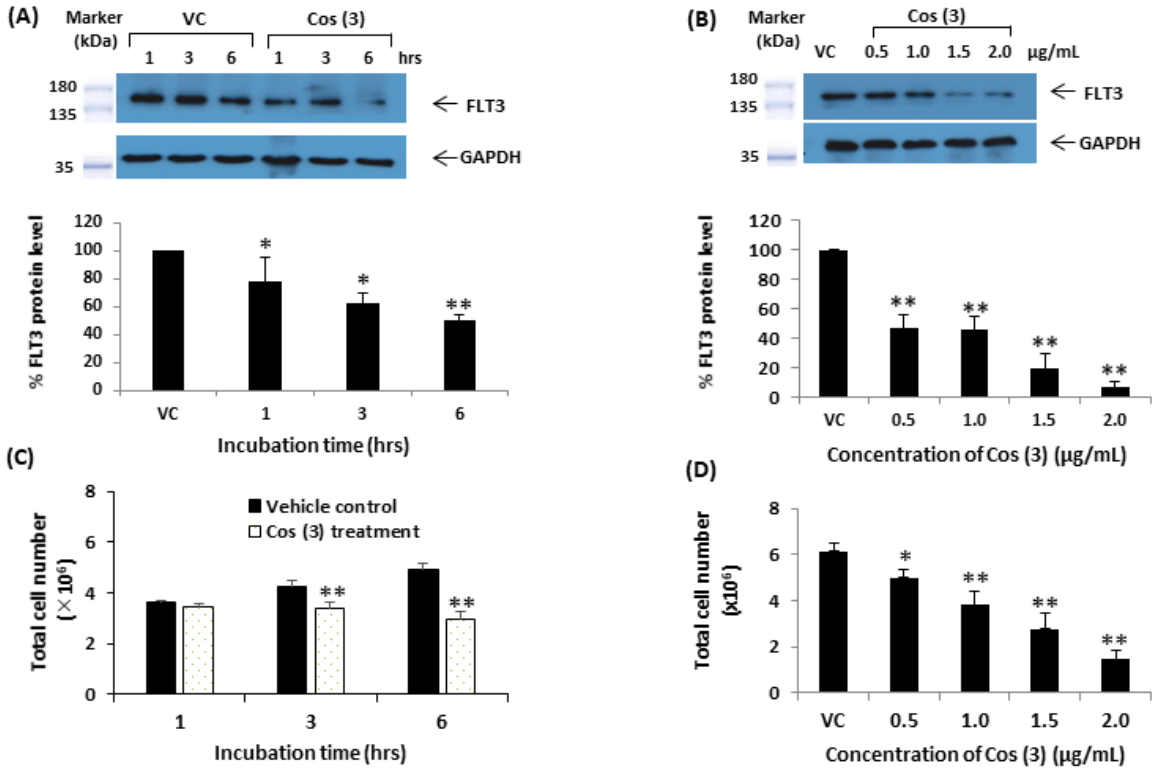

Figure 7. *Effect of various times and concentrations of standard costunolide (Cos (3)) on FLT3 protein expression in EoL-1 cells by Western blot analysis. (A) EoL-1 cells were cultured with Cos (3) at the concentration of 1.0 µg/mL for 1, 3, and 6 hrs. (B) EoL-1 cells were cultured with different concentrations of Cos (3) (0.5, 1.0, 1.5, and 2.0 µg/mL) for 6 hrs. FLT3 protein expression level was measured by Western blotting and GAPDH was used as loading control. (A and B) Densitometry was used to quantitate the protein levels as the percentage of vehicle control (0.02% DMSO alone without the Cos (3) in culture medium). (C and D) The total cell numbers were measured by trypan blue exclusion method. Data are the mean ± SD of three independent experiments. Asterisk (*) (**) denotes a significant difference from the control group ($p<0.05$ and $p<0.01$).*

Discussion

Magnolia consists of about 240 species throughout the world[22] with 112 species distributed in tropical and subtropical parts of Asia. In Thailand, 25 species are found.[23] It has been used in a number of traditional medicine preparations in China and Japan. Magnolia is a rich source of several biological active compounds. It was reported to have at least 255 different ingredients, such as alkaloids, coumarins, flavonoids, lignans, neolignans, phenylpropanoids, and terpenoids.[24] Among these, several neolignan ingredients including magnolol, honokiol, 4-o-methylhonokiol, and obovatol have been the focus of studies examining various pharmacological effects of Magnolia. Champi Sirindhorn is a new species of *Magnolia spp*.[10] leaves of *M. sirindhorniae* consist of five sesquiterpene lactones such as costunolide, santamarine, reynosin, parthenolide, and lipiferolide.[10] This study is the first report to examine the effect of *M. sirindhorniae* extracts on FLT3 protein expression in leukemic cells. Nine crude fractional extracts (fraction No.1-9) from leaves, twigs, and stems were analyzed for their ability to inhibit leukemic cell growth under the FLT3 protein suppression which is involved in human leukemic cell proliferation and compared their activity to purified active compounds (costunolide (1) and parthenolide (2)). Furthermore, commercial costunolide (3) and parthenolide (4) were also determined to compare to those of purified compounds. EoL-1 cells were selected to furnish the analysis via the FLT3 overexpressing cell model due to high levels of FLT3 protein. Cytotoxic effects of crude fractional extracts (Hex, EtOAC, and MeOH) from leaves, twigs, and stems of *M. sirindhorniae* were assessed by MTT assay. Only crude fraction from Hex and EtOAC showed cytotoxic effects on EoL-1 cells. Furthermore, fraction No.7 (stems/ Hex) showed the strongest cytotoxicity in EoL-1 cells.

Effects of fraction No.1-9 (IC_{20} values) on FLT3 protein expression were examined by Western blotting. We found that extraction No.1 (leaves/ Hex) that has the highest

costunolide content in all fractions (7.0%) showed strong inhibitory effects on FLT3 protein expressions in EoL-1 cells. Concentration at IC_{20} value of fraction No.1 treatment was 20 µg/mL. The results showed the FLT3 suppression by a time- and dose- dependent manner after fraction No.1 treatment. In addition, extraction No.5 (twigs/ EtOAc) which has the highest parthenolide content in all fractions (39.6%) at concentration of 29 µg/mL showed the strongest inhibitory effects on FLT3 protein expressions in EoL-1 cells. The results also showed the FLT3 suppression by a time- and dose- dependent manner after fraction No.5 treatment. Moreover, 3.9 µg/mL of costunolide (1) which has the costunolide content (49.6%) decreased FLT3 protein expressions in EoL-1 by 66.0%. Furthermore, it decreased FLT3 protein by a time- and dose- dependent manner. In addition, 1.0 µg/mL of parthenolide (2) which has the parthenolide content (78.4%) decreased FLT3 protein expressions in EoL-1 by 78.3%. However, the effect of costunolide (1) to suppress FLT3 protein expression was not significantly difference when compare with parthenolide (2). Importantly, 0.9 µg/mL of the commercial costunolide (3) and 0.8 µg/mL of parthenolide (4) with the costunolide contents ≥97% and parthenolide ≥98%, respectively by HPLC decreased FLT3 protein expressions in EoL-1 cells by 91.5% and 90.4%, respectively. Taken together, it revealed that extraction No.1, 5, purified active compounds (costunolide (1), and parthenolide (2)) significantly inhibited cell proliferation without cell viability alteration via the suppression of FLT3 protein expression at non-cytotoxic doses in EoL-1 cells.

The effective compounds costunolide (1) and parthenolide (2) of *M. sirindhorniae* need further studies. The leaves extract of *M. sirindhorniae* has been reported the cytotoxicity.[10] Costunolide, parthenolide, and lipiferolide demonstrated more cytotoxicity against human breast cancer cell line (IC_{50} values of 2.17-5.24 µg/mL) than the standard drugs doxorubicin (IC_{50} value of 9.04 µg/mL) and tamoxifen (IC_{50} value of 9.61 µg/mL). In addition, the parthenolide (major compound) showed the highest activity against human epidermoid carcinoma (KB), human breast cancer (MCF7), and human small cell lung cancer cells (NCI-H187) with the IC_{50} values of 1.67, 2.17, and 0.97 µg/mL, respectively.[10] Furthermore, the cytotoxicity of Hex fraction from *M. siamensis* flower extract against EoL-1 cells has been reported with the IC_{50} value of 3.8 µg/mL[25] and showed 1.4- and 1.0- fold stronger than in costunolide (1) and parthenolide (2), respectively. However, costunolide (3) and parthenolide (4) showed 3.4- and 1.8- fold, respectively stronger than in Hex fraction from *M. siamensis* flower extract. Curcumin is the well-known FLT3 inhibitor. The cytotoxicity (IC_{50} value) of curcumin against EoL-1 cells was 6.7 µM (2.5 µg/mL)[26] which showed 2.2- and 1.6-fold stronger than costunolide (1) and parthenolide (2), respectively, whereas costunolide (3) and parthenolide (4) showed 2.3- and 1.2- fold, respectively stronger than that of curcumin. We found that, the inhibitory effect of costunolide (1) and parthenolide (2) at the concentration of 3.9 and 1.0 µg/mL, respectively (IC_{20} value) on the suppression of FLT3 protein expression in EoL-1 cells were 3- and 4-fold stronger than 1.7 µg/mL (IC_{20} value) from Hex fraction of *M. siamensis* flower extracts (which could suppress the FLT3 protein expression by 21.1%)[25] when compared at the IC_{20} values. Moreover, the IC_{20} values of costunolide (1) and parthenolide (2) could suppress FLT3 protein expression in EoL-1 cells were 5.5- and 6.5-fold stronger than 4.0 µM (1.5 µg/mL) (IC_{20} value) of curcumin.[26]

This study is the first report to show that *M. sirindhorniae* extracts demonstrate their inhibitory effects on FLT3 protein in leukemic cells. These results suggested that the active compounds costunolide (1) and parthenolide (2) of *M. sirindhorniae* inhibited FLT3 target protein related to leukemic cell proliferation, and thus can potentially be used for developing new anti-cancer drugs.

Conclusion

M. sirindhorniae is a source of costunolide (1) and parthenolide (2), the active compounds responsible for the inhibition of FLT3 protein expression in leukemic cells. The major sources of costunolide (1) and parthenolide (2) are from the leaves and twigs, respectively. This is the first report of the inhibitory effect of costunolide (1) and parthenolide (2) on FLT3 protein expressions in leukemic cells. The results were in parallel with the commercial costunolide (3) and parthenolide (4). In addition, costunolide (1) and parthenolide (2) showed the high activity for FLT3 protein suppression in EoL-1 cells. This natural product displays a potent inhibitory activity on leukemic cell proliferation and may have therapeutic potential as an anti-leukemic drug. Hence, we suggest that costunolide and parthenolide are promising compounds and can be successfully exploited in leukemic drug in future. Moreover, traditional herbal medicine industry is also the target for herbal drug product as an alternative for leukemia patients.

Conflicts of interests

There are no conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Active compounds, free radicals scavenging and tumor-necrosis factor (TNF-α) inhibitory activities of star fruit-sweet type (*Averrhoa carambola L.*) *in vitro*

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ABSTRACT

Background: Star fruit (*Averrhoa carambola L.*) is a seasonal fruit that originated in Southeast Asia, including Thailand. There are two distinct types; sour and sweet, with various active compounds such as saponins, flavonoid C-glycosides, tannin, L-ascorbic acid, (-) epicatechin and gallic acid. However, the active compounds, especially total phenolic compound of star fruit-sweet type, have not been fully investigated.

Objectives: Thus, this study aimed to investigate the active compounds, radical scavenging and anti-inflammatory activity of star fruit-sweet type *in vitro*.

Materials and methods: Total phenolic compound, total flavonoids and L-ascorbic acid in extract were evaluated by Folin-Ciocalteau reagent, aluminum chloride colorimetric assay and high-performance liquid chromatography, respectively. Radical scavenging activity on 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) cation radicles and nitric oxide (NO), and protective activity of glutathione (GSH) from free radicals generated by high voltage-stimulation in plasma micro/nano bubble water were studied. Finally, the anti-inflammatory activity on tumor necrosis factor-alpha (TNF-α) release was performed in lipopolysaccharide (LPS)-activated macrophage cells.

Results: One hundred grams star fruit extract showed total phenolic compounds as 5.12±0.24 µg of gallic acid, total flavonoids as 0.185±0.008 µg of quercetin and 5.24±0.55 µg of ascorbic acid. One gram of Star fruit extract, equal to 722.71±12.25 µg of gallic acid (GAE), showed radical scavenging activity on $ABTS^{\bullet+}$. Star fruit extract (27.48±1.8 µg of IC_{50}), standard gallic (24.28±2.6 µg of IC_{50}) and ascorbic acid (54.98±2.5 µg of IC_{50}) also showed radical scavenging activity on NO, respectively. Whereas Star fruit extract also scavenged free radicals generated by 10 min of high voltage (HV) stimulation in micro/nano-bubble water, and protected GSH with

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dose response (125-1,000 µg/mL), in the same way as standard ascorbic acid. Finally, the star fruit extract, and also standard hydrocortisone, had anti-TNF-α releases with dose responses.

Conclusion: The star fruit extract-sweet type has antioxidant activity with phenolic compound, flavonoids, and ascorbic acid, as well as having anti-inflammatory activity.

Introduction

Many fruits are available in Thailand for all-year consumption such as banana, pomelo, papaya, grape, and mango, as well as seasonal fruits such as rambutan and custard apple. These fruits contain various multi-vitamins and polyphenolic compounds with antioxidant activity that benefits to human health.[1]

Star fruit or Carambola is a rare seasonal fruit found locally in Thailand. Its scientific name is *Averrhoa carambola* Less. It has been cultivated in tropical areas of Southeast Asia for many centuries. Nowadays, star fruit has many species or varieties such as Taiwan (large size with a green edge and sweet taste), Malaysia (large size with a sour-sweet taste, and much juice), and Guangdong, China (large size and white with a sweet taste).[2] However, previous study has reported two distinct classes of carambola. Firstly, the smaller type of star fruit has a very sour and rich flavor, with more oxalic acid than other types, which are larger, and have a rather bland mildly sweet flavor as well as oxalic acid.[2] Secondly, the folk variety in Thailand is small with the typical characteristic of a five-pointed star-like cross section. It has a green to yellowish skin, and very sour-slightly sweet flavor.[3] Previous reviews showed its chemical constituents as saponins, flavonoid C-glycosides, tannin,[4,5] L-ascorbic acid, (-) epicatechin and gallic acid,[6,7] as well as nutritional values that can be used in traditional medicine, for example, anti-pyretic drugs, laxatives, appetite stimulants, diuretics and digestives.[2,7] A previous study of star fruit juice supplement showed the folk variety in Chiang Mai province as having antioxidant activity with a high level of L-ascorbic acid (Vit C) (approximately 16-17 mg in 100 g of extract) and low retinoic acid (approximately 0.1-0.2 µg in 100 g of extract). In addition, when a supplement of fresh ripe star fruit juice was administered to healthy elderly people at 100 g for one month, high density lipoprotein (HDL) increased and low density of lipoprotein (LDL) decreased significantly.[8] Moreover, it reduced inflammatory conditions by reducing plasma TNF-α, IL-23 and nitric oxide (NO) levels.[9] Unfortunately, the folk variety has become almost extinct, due to being less popular among farmers and consumers because of its very sour taste. Nowadays, large and sweet types of star fruit are available such as the previously mentioned Malaysia type and varieties from India, which are cultivated and distributed in many markets in Thailand. Therefore, this study aimed to recheck their active compounds, especially total phenolic compound, total flavonoids, L-ascorbic acid, and scavenging activity on radicals such as organic cation radicals, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) $(ABTS)^{\bullet+}$ and NO. Moreover, the activity of protecting glutathione (GSH) oxidation from radicals generated by high voltage activation in a micro/nano bubble water model. The inhibition effect on inflammatory cytokine release in macrophage cell lines was confirmed in this study.

Materials and methods

Star fruit preparation

The Malaysia variety of raw star fruit, cultivated at organic gardens in Pathum Thani province, was purchased for this study. All of the star fruit was baked in boxes for 2 weeks until ripe (Figure 1) and then cleaned by soaking in clean water five times before blending in a fine homogenizer. Fibers and seeds were removed by filtering through clean filter cloth, and the star fruit juice was kept in a clean bottle before being produced in dry powder form, or crude extract by freeze drying technique at the Argo-Industrial Business Service Center, Faculty of Agro-Industry, Chiang Mai University, Thailand. Final yield of crude extracts from fresh star fruit (5.33%/*w:w*) was collected in a dark bottle and refrigerated before analysis.

Figure 1. *Ripe, Sweet Star Fruit.*

Active Compound Analysis

Total phenol

Total content of phenol in star fruit extract was determined by Singleton and Rossi method (1965),[10] in which 40 µL of extract (25 mg/mL) was mixed with 1.8 mL of diluted Folin-Ciocalteau reagent (10% v/v) (Merck KGaA, Germany), and kept in dark for 5 min before 1,200 µL of (7.5%) sodium carbonate (Merck, Dermstadt) was added. After that, the tubes were incubated for 60 min, and pellets removed by centrifugation at a speed of 10,000 rpm for 3 min, with the supernatant being read at 765 nm by spectrophotometry (Drawell Scientific, Shanghai). Total phenolic content was calculated compared to standard gallic acid (0.008-1.0 mg/mL) (Fluka, Switzerland).

Total flavonoid content

Total flavonoid content was measured using aluminum chloride colorimetric assay, adapted from a previous protocol.[11] Aqueous star fruit extract at 2.5 mg/mL or 500 µL of different dilutions of standard quercetin solution (0.078-2.5 mg/mL)

(Aldrich, Germany) were added in 100 µL of 10% $AlCl_3$ (Fischer Scientific, UK). Then, 100 µL of sodium acetate (1.0 mol/L) (Fischer Scientific, UK) was added to 2.8 mL of deionized water. After incubation with light protection for 30 min at room temperature, absorbance was measured by spectrophotometry (Drawell Scientific, Shanghai) against a freshly prepared reagent blank at 415 nm. Total flavonoid content of the extract was expressed as mg of quercetin (Sigma-Aldrich, Germany); equivalent to one gram of extract.

L-ascorbic acid assay

The protocol for evaluating the ascorbic acid content in star fruit extract was performed by high-liquid chromatography (HPLC) as previous described.12 Before analysis, 20 mg of extract was dissolved in 1.0 mL of deionized water. Precipitate was removed by centrifugation (10,000 rpm for 10 min in a cool room), and the supernatant was filtered (0.22 µm). The analysis was performed in the experimental condition using a C18 reverse phase column (Eclipse Plus C18: 5 µm, 4.6 x 250 mm; Agilent, USA) under formic acid (0.1% v/v) (Sigma-Aldrich, Germany) as a mobile phase (pH 2.5). The flow rate was 0.8 mL/min and injection volume 20 µL for all of the analyses. Total run time was 25 min and retention time for peak ascorbic acid 3.91-3.93 min, identified by a diode array detector (DAD) (SPD-M20A, SHIMADZU, JAPAN) at 244 nm. Concentration of L-ascorbic acid in extract was compared to standard L-ascorbic acid (Fisher Scientific, UK).

Scavenging activity assays

Scavenging activity of extract on bleach, 2,2-Azino-bis (3-ethylbenzo thiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), followed previous protocol.[13] $ABTS^{\bullet+}$ was produced by reacting ABTS (CALBIOCHEM, Darmst adt, Germany) solution (14 mmol/L) with 14 mmol/L of potassium persulfate (Merck KGaA, Darmstadt, Germany) in deionized water for 12 hrs in dark. $ABTS^{\bullet+}$ stock was diluted in phosphate buffered solution (PBS) in order to start an absorbance of 0.70±0.02 at 734 nm by spectrophotometry (Drawell Scientific, Shanghai). Ten microliter of extract or standard Trolox (Aldrich, Germany) (0-10 mmol/L) was added in 990 µL of working $ABTS^{\bullet+}$ solution in a plastic cuvette (size 1.5 mL), and gently alternated inversely 5 times before absorbance was read. The percentage decrease of absorbance by spectrophotometry was calculated as follows: (AbsBlank-Absample) x 100/AbsBlank. All of the tests were evaluated three times and averaged. Scavenging activity result of extract at one gram was represented by comparing with the standard gallic acid (µg) (Fluka, Switzerland).

Nitric oxide (NO) scavenging assay

NO radical inhibition was adapted from a previous protocol.[14] NO was generated by dissolving sodium nitroprusside (10 mmol/L) (AnalaR NORMAPUR, VWR, Prolabo, Belgium) in deionized water, and kept in light at room temperature for 3 hrs before study. The reaction mixture (3 mL), containing 2 mL of (10 mmol/L) sodium nitroprusside (SNP), 0.5 mL of saline phosphate buffer that contained KH_2PO_4, Na_2HPO_4, NaCl and KCl (Merck, USA) (pH.7.4), 0.5 mL of standard gallic acid (Fluka, Switzerland) solution, and ascorbic acid (Fisher Scientific, UK) at 0.78-200 µg/mL or aqueous extracts (500–1,000 µg/mL), was incubated at 25°C for 150 min. After incubation, 0.5 mL of the reaction mixture was mixed with 1.0 mL of sulfanilamide (Fluka, China) (1% in 2.5% of H_3PO_4, Merck, USA) and allowed to stand for 5 min. After completion, a further 1 mL of napthyl ethylene diamine dihydrochloride (0.1% in water) (VWR, Prolabo, Belgium) was added, mixed, and allowed to stand for 20 min at 25°C. Then, absorbance was read at 537 nm by spectrophotometry (Drawell Scientific, Shanghai). Percentage of inhibitory or scavenging activity was calculated using the following formula:

$$\% \text{ inhibitory or scavenging activity} = \frac{[(A_{control} - A_{test} \text{ or } A_{std})]}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of control, A_{test} and A_{std} are the absorbance of test and standard, respectively. The results presented the half-maximal inhibitory concentration (IC_{50}) between extract and standard antioxidants; L-ascorbic acid and gallic acid.

Protective activity of glutathione (GSH) oxidation from free radicals

A new model to estimate the protective activity of GSH from free radicals by starting fruit extract consumption was designed with high-voltage (HV) stimulation in micro/nano-bubble (mnb) water mixture or Plasma-nano bubble technique at the High Voltage Engineering Laboratory, Department of Electrical Engineering, Faculty of Engineering, Rajamangala University of Technology Lanna, Chaing Mai, Thailand.[15] Several research works have demonstrated that discharged plasma in ionized water is able to dissociate water molecules that lead to production of many reactive species such as radicals (hydroxyl radicals, $OH^{\bullet}$; superoxide radical, $O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), etc.[16] Then, GSH can be oxidized directly by those radicals in the system,[17] when electrically discharged plasma is in the water. Laboratory-made plasma generator in this model consisted of an HV power supply and a discharged plasma electrode. Micro/nano-air bubble water was generated in deionized water by a micro-bubble generator (AURA Tec Co., Ltd., model OM4-MDG-045) before preparing GSH stock (Sigma, St. Louis, Co, USA) at 100 mg/mL (Figure 2A-2C). One hundred mL of GSH stock solution was prepared in a 150-mL beaker before standing in a plastic box (Figure 2D). HV power supply used a high voltage transformer and direct current (DC) half wave circuit to convert input current at 1.5-2.0 amps, 100 volts and 50 Hz into an HV of up to 6 kVp and 1 Ap of discharged current. The discharged plasma electrodes had a ground electrode placed at the bottom of the beaker, and an anode electrode of tungsten (1.5 mm diameter) was dipped into the solution to produce discharged electrical plasma

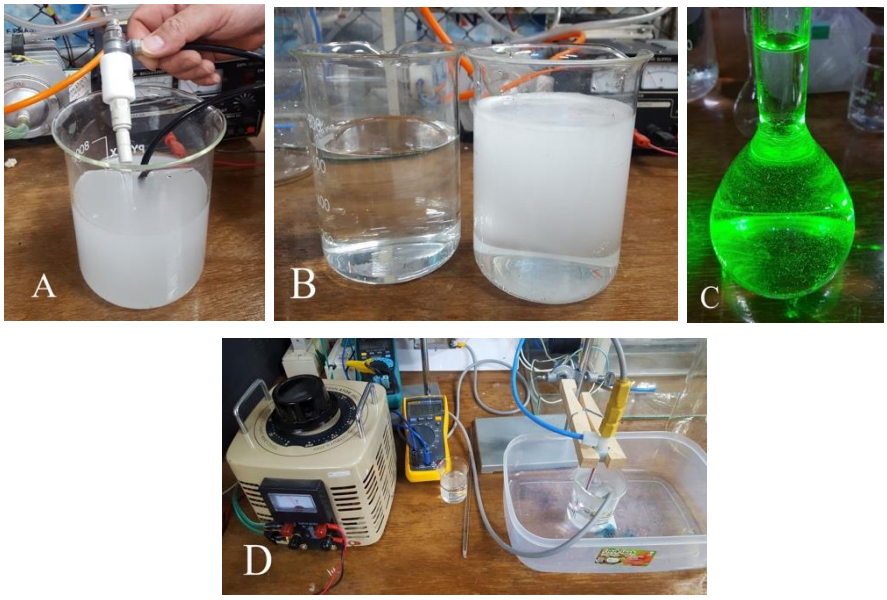

***Figure 2**. High-voltage stimulation in micro/nano-bubble water. Micro/nano-bubble (A), when compared to non-micro/nano bubble water (B), scattering light of micro/nano-bubble in water (C), and high-voltage stimulation in the micro/nano-bubble water system (D).*

Ten min after starting the system, free radicals were generated and GSH oxidized in the system with and without 125-1,000 µg/mL of star fruit extract. Residual GSH concentration was determined using the 5, 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) protocol.[9] Two hundred microliter of mixed solution was taken to mix with 500 µL of DTNB (Sigma-Aldrich, Germany) and 500 µL of phosphate buffer (pH 8.0) solution. After incubating at room temperature for 5 min, a clear yellow supernatant solution was read by spectrophotometry at 412 nm (Drawell Scientific, Shanghai). Percentage of GSH was presented compared to non-HV stimulation and standard ascorbic acid (Fisher Scientific, UK) at 100 and 200 µg/mL, respectively.

Anti-inflammatory response protocol

Before evaluating the inhibitory activity in cells, viability of J774A.1 mouse macrophage culture was determined by 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay.[18] Briefly, J774A.1 cells (10^6/mL) were seeded for 4 hrs in a 96-well plate in the presence of star fruit extract (50-1,600 µg/mL), and with or without lipopolysaccharide (LPS) in the final volume of 200 µL. Medium was removed after 24 hrs, with 20 µL of MTT solution (5 mg/mL) (Sigma-Aldrich, St.Louis, MO, USA) being added to each well for 4 hrs. The resulting formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Then, optical density (OD) was measured on a microplate reader at 570 nm. Percentage viability was calculated using the formula: OD of treated cells/OD of corresponding control × 100.

Concentrations of star fruit extract, with a cell viability of more than 90%, were used to evaluate the effect on tumor-necrosis factor-alpha (TNF-α) production by activating macrophages. J774A.1 cells were kindly supplied by Dr. Penpitcha Wanachantararak (Dental Research Center, Faculty of Dentistry, Chiang Mai University, Thailand). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of heat-inactivated fetal bovine serum (FBS, Gibco, BRL), 100 µg/mL of streptomycin and 100 U/mL of penicillin (Sigma-Aldrich, St.Louis, MO, USA) and incubated at 37°C in a humidified atmosphere containing 5% CO_2.[19] Cells were grown to confluence in sterile culture flasks, counted by a haemocytometer and seeded in triplicate at a density of 1×10^5 cells/mL in a 6-well flat-bottomed tissue culture plate in the presence of LPS (1 µg/mL) (Sigma-Aldrich, St.Louis, MO, USA), for 24 hrs at 37 °C. Then, various concentrations of star fruit extract (62.5, 125 and 250 µg/mL) were compared with standard hydrocortisone (50 ng/mL) and added to the culture simultaneously with 1 µg/mL of LPS. Cells were incubated at 37°C for 24 hrs. After completion, supernatants were collected after centrifugation, and TNF-α was determined by a specific Quantikine ELISA-kit (R&D systems, USA).

Statistical analysis

All data were represented with a mean and standard error of mean (SEM). One-way ANOVA was used for statistical analysis between standard antioxidants and extracts.

Results

The results of active compounds

One hundred grams of star fruit extract presented the active compounds (Table 1), with total phenolic compound being equivalent to 5.12±0.24 µg of standard gallic acid, while total flavonoids were equivalent to standard quercetin at 0.18±0.008 µg. In analyzing ascorbic acid in 30 mg/mL of star fruit extract, at a peak of 3.91 min (Figure 3.A), the retention time was similar to that of standard L-ascorbic acid (3.901 min) (Figure 3.C). When compared with the dose response (R=0.997, p=0.003) of standard L-ascorbic acid from the predicted equation (Figure 3.B), the yield of ascorbic acid in 100 grams of extract was equal to 5.24±0.5 mg of standard L-ascorbic acid.

Table 1 Active compounds of star fruit extract (100 gms).

Active compounds	Concentration
Total phenolics	5.12±0.24 µg Gallic Acid Equivalent (GAE)
Total flavonoids	0.18±0.008 µg Quercetin Equivalent (QE)
Ascorbic acid	5.24±0.5 mg

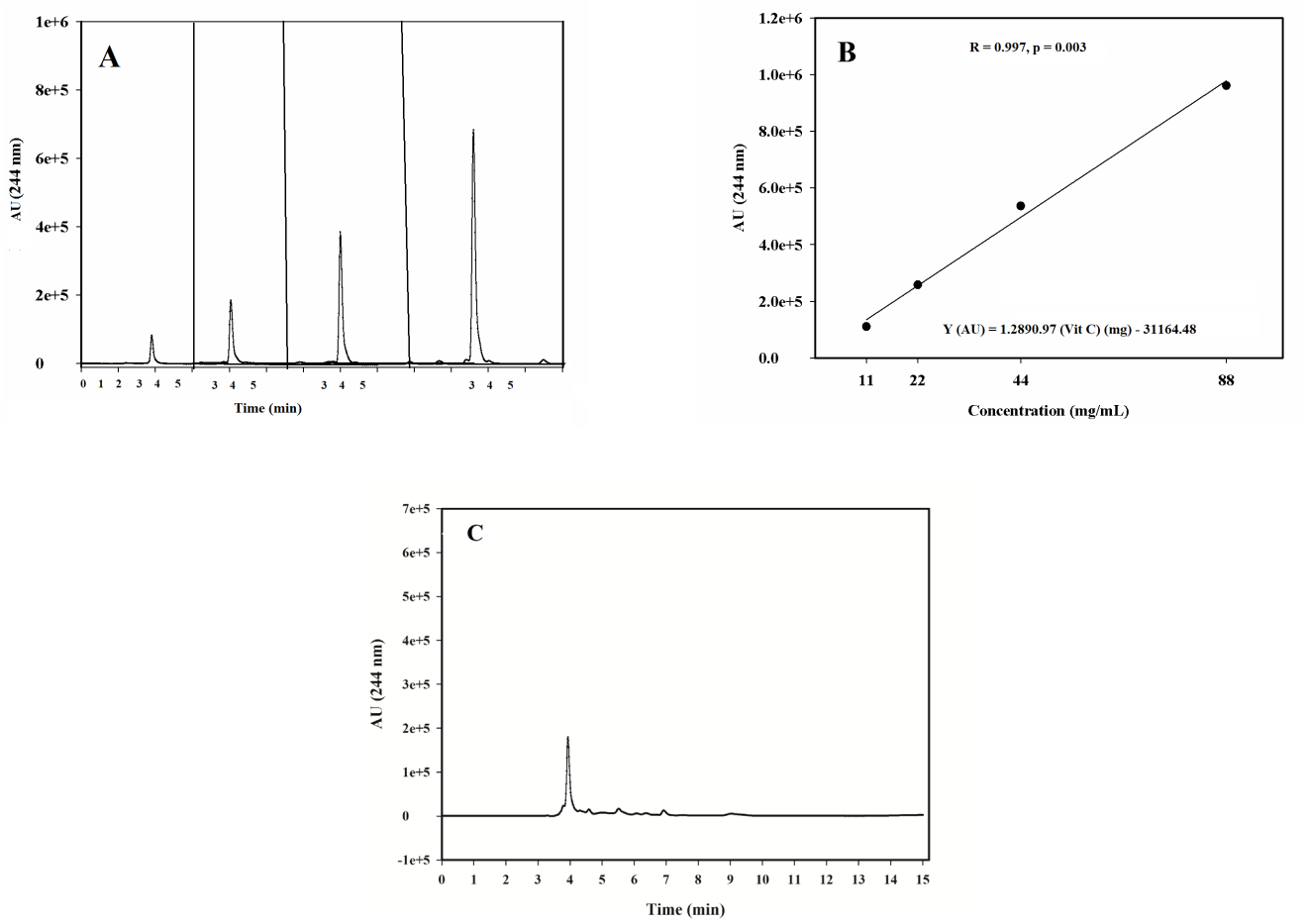

Figure 3. *HPLC peaks of standard ascorbic acid at 11-88 mg (A), standard curve of standard ascorbic acid at 11-88 mg (B), and peak of star fruit extract at 3.91 min (C). Y-axis is an absorbance unit (AU).*

Scavenging activity

The models for evaluating radical scavenging were actioned by using two systems for scavenging ABTS cation radicals and NO. The results in Table 2 showed that the comparison of maximal extract activity of standard gallic acid at one gram was 722.71±12.25 µg on scavenging the ABTS cation radical. For inhibitory activity on NO at approximately 190 µmol/L was generated continuously after being kept in room temperature for 3 h (Figure 4A). Then, star fruit extracts were added to SNP solution at different concentrations and kept in a cold room at 25°C for 150 min. The activity of star fruit extract scavenging on NO was compared to that of standard gallic and ascorbic acid by an inhibitory concentration of 50% (IC_{50}). The results showed an exponential rise to maximum with dose response (Figure 4B), and the IC_{50} of star fruit extract was 27.48±1.8 µg when compared to that of standard gallic (24.28±2.6 µg) and standard ascorbic acid (54.98±2.5 µg).

Table 2 Scavenging activity of star fruit extract.

Scavenging activity	Concentration
ABTS radical	722.71±12.25 µg GAE/gm extract
Nitric Oxide (IC_{50})	
- Std. ascorbic acid	54.98±2.5 µg
- Std. gallic acid	24.28±2.6 µg
- Star fruit extract	27.48±1.8 µg extract

Note: GAE: Gallic Acid Equivalent, IC_{50}: Inhibitory Concentration at 50%

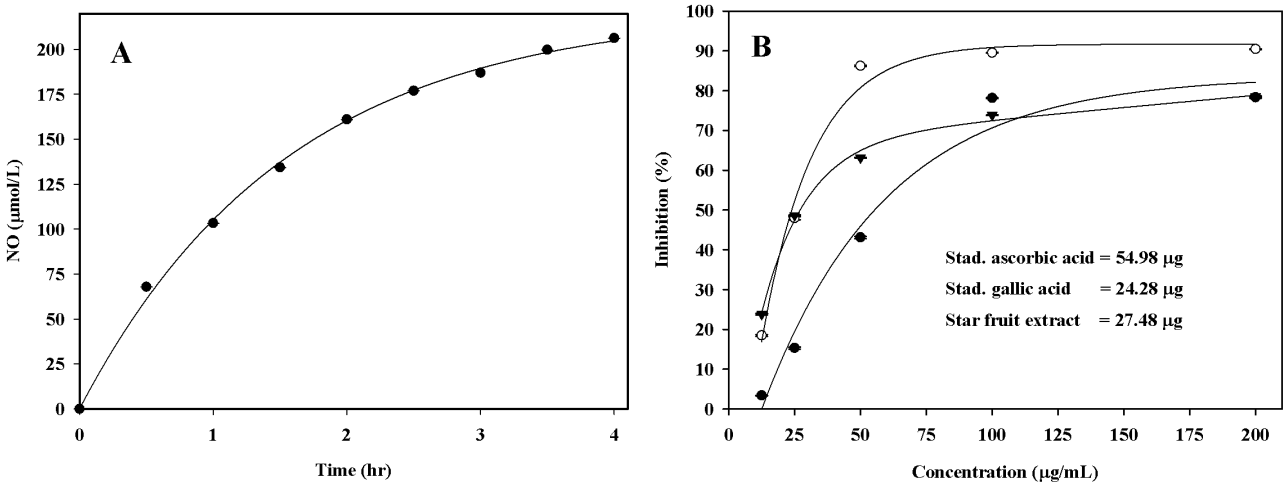

Figure 4. *NO production from SNP at time responses (A), percentage of inhibition or NO scavenging between star fruit extract, and standard ascorbic and gallic acid (B). IC50 = inhibitory concentration at 50%.*

Protective activity of glutathione (GSH) oxidation from free radicals

Protective activity of GSH from free radicals, generated by HV and stimulated by micro/nano-bubble technique, was studied. HV system, stimulated by micro/nano-bubble water containing 100 mg/mL of GSH, showed that GSH was oxidized serially in responding to 5-20 minutes of incubation time. GSH was oxidized at 100 mg/mL and remained in the system at 39.92±0.31, 28.9±0.05, 20.8±0.01 and 12.40±0.13% after 5, 10, 15, and 20 min, respectively (Figure 5.A). When compare to 10 min of HV stimulation, GSH decreased significantly to 32.33±0.05%. After adding standard ascorbic acid (vit C) at 100 and 200 µg/mL, the GSH concentration remained at 65.23±1.5 and 80.43±1.61%, respectively, therefore, the standard ascorbic acid showed significant protective activity when compared to the system. It was interesting that significant protective activity with dose responses was shown when star fruit extract was added at 1,000-125 µg/mL (71.84±2.85, 62.38±1.50, 57.32±1.05 and 42.45±1.11% of GSH) (Figure 5B).

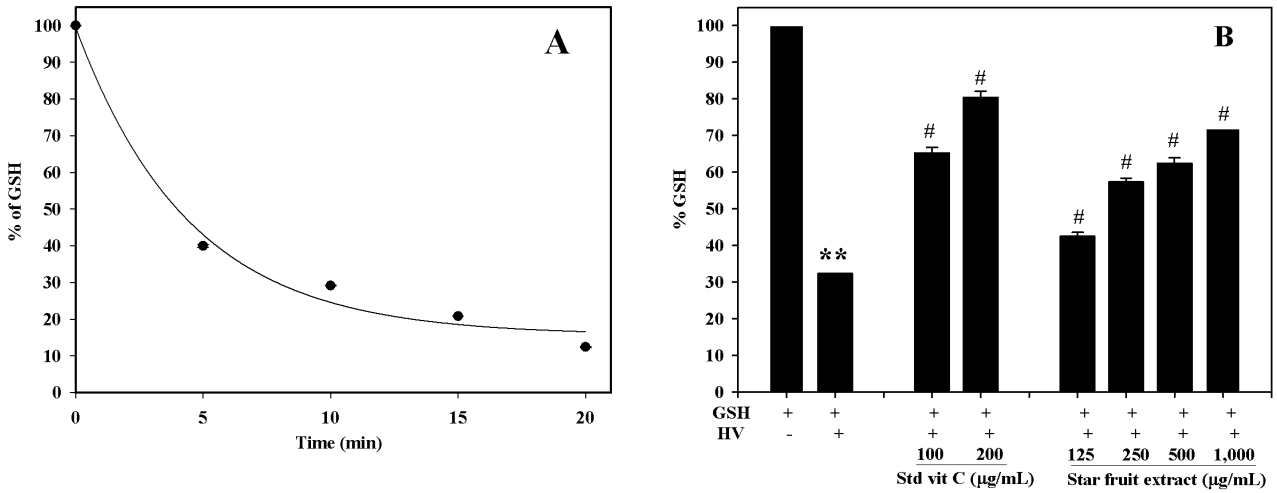

Figure 5. *Percentage of GSH after free radical-generated oxidation by high voltage stimulation in the micro/nano bubble (mnb) water system for 5-20 min (A), potential protective activities of standard ascorbic acid (vit C) at 100, 200 µg/mL and star fruit extract at 125-1,000 µg/mL (final concentration) (B). HV: high voltage stimulation, **p<0.01 when compared to GSH without HV stimulation, #p<0.01 when compared to GSH with HV stimulation (Mann-Whitney U Test).*

Inhibitory activity of TNF-α in macrophages

Anti-TNF-α released from LPS-activated macrophage cell line (J774A.1) in the experimental study showed the series response of star fruit extract at 62.5, 125, and 250 µg/mL, when compared to LPS-activated macrophage cells (Figure 6). Results showed significantly dominant TNF-α levels released from macrophage cells after activation with LPS for 24 hr (56.10±2.5 pg/mL). After incubating with extracts at 62.5 µg/mL, TNF-α showed a significant decrease to 52.62±2.3 pg/mL after 24-hrs incubation, and more significant inhibitory activity was found at the higher concentrations of 125 µg/mL (48.75 ±1.3 pg/mL) and 250 µg/mL (42.0±2.10 pg/mL). In addition, cells treated with 50 ng/mL of hydrocortisone (a standard inflammatory depressor) showed a significant depression in TNF-α release (12.5±3.2 pg/mL).

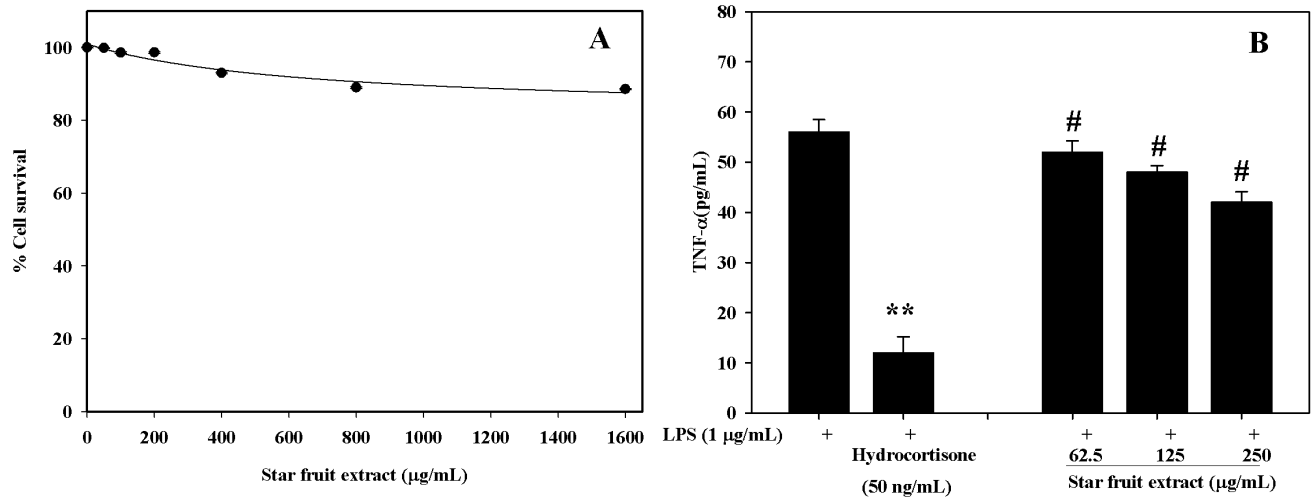

Figure 6. *Percentage of cell survival after incubation with star fruit extracts at different doses (50-1,600 µg/mL) (A), and TNF-α level between standard hydrocortisone (50 ng/mL) and extracts at 62.5-250 µg/mL) (B). **p<0.01 and #p<0.05 (Mann-Whitney U Test).*

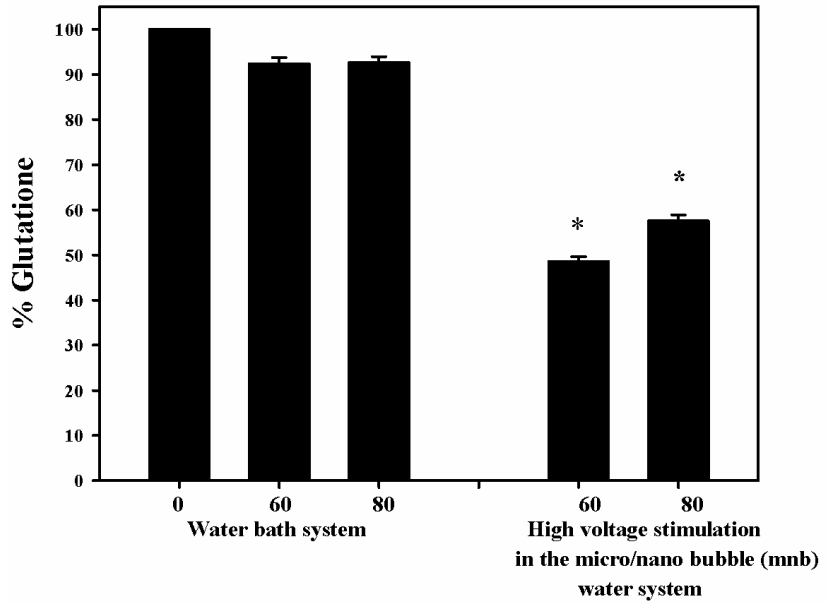

Figure 7. *Percentage of glutathione (GSH) between the water bath system and high voltage stimulation in the micro/nano bubble (mnb) water system at 60 and 80 degrees Celsius. *p<0.05 (Wilcoxon Sign Rank Test).*

Discussion

This study was a preliminary work on the sweet type of star fruit distributed in Thailand. Previous evidence showed that the active compounds in star fruit were saponins, flavonoid C-glycosides and tannin,[4,5] as well as L-ascorbic acid (-), epicatechin and gallic acid.[6,7] In particular, the folk or sour type in Chiang Mai province, Thailand, contained approximately 16-17 mg of L-ascorbic acid in 100 g of extract.[8] However, yield of L-ascorbic acid in sweet-type star fruit was approximately 5-6 mg in 100 g of extract. Nevertheless, this study found total phenolic compound, total flavonoids and L-ascorbic acid in other types of sweet star fruit distributed in Thailand.

Moreover, this study proved the activity of extract on scavenging radicals with different modes; $ABTS^{\bullet+}$, NO, and free radicals generated by the micro/nano-bubble water system. ABTS cation radicals were used in general system to estimate total antioxidant capacity (TAC) of natural products by comparing with standard Trolox.[21] Protocol for scavenging $ABTS^{\bullet+}$ was modified by estimating maximal extract scavenging for at least 10 min. A previous study suggested that $ABTS^{\bullet+}$ assay could be measured within 2-10 min.[22] Therefore, the maximal scavenging activity of extract or standard Trolox was observed on stable or plateau absorbance, although there is no evidence on the sweet-type star fruit, total phenolic compounds, total flavonoids or L-ascorbic acid found in this study. This result might be consistent with a previous study that found antioxidant capacity of star fruit on scavenging $ABTS^{\bullet+}$.[5] In addition, star fruit contains flavonoids and non-phenol compounds [diglucosides, carambolasides, four phenyl-propanoids; (+)-isolariciresinol 9-*O*-β-D-glucoside, (+)-lyoniresinol 9-*O*-β-D-glucoside, (−)-lyoniresinol 9-*O*-β-D-glucoside and 1-*O*-feruloyl-β-D-glucose, three benzoic acids, protocatechuic acid and 1-*O*-vanilloyl-β-D-glucose].[23]

Furthermore, another model on scavenging NO also was studied, due to previous results in a study on the elderly, in which plasma NO reduced after consumption of star fruit juice for 4 weeks.[9] NO scavenging in star fruit extract was performed in this study by following the previous protocol.[14] NO was generated from SNP in deionized water. The protocol showed that NO was generated continuously and reached a plateau after being kept in light at room temperature for 3 hr. Therefore, after SNP was dissolved for 3 hr, the scavenging activity of extract was evaluated. The results confirmed the previous study of Leelarungrayub and co-workers in that star fruit sour-type could reduce the NO in elderly people because of its scavenging activity.[9] Gallic and L-ascorbic acid were used in the model to compare with star fruit extract. Previous evidence confirmed that gallic acid was a versatile scavenger that rapidly deactivated a wide variety of reactive oxygen species (ROS) and reactive nitrogen species (RNS).[24] This study found that scavenging activity of star fruit extract (27.48±1.8 µg) on NO was similar to that of standard gallic acid (24.28±2.6 µg), thus, the active compounds in star fruit extract, like phenolic compounds, can be preferred. This study also found that both total phenolic and flavonoid compounds in star fruit extract might relate to their antioxidant capacity in scavenging $ABTS^{\bullet+}$ and NO. The correlation between total phenolic compound and total flavonoids on antioxidant capacity is consistent with a previous report.[25]

Moreover, this study evaluated the scavenging activity of star fruit extract with a new model: Micro/nano-bubble water system, which has been known as Plasma-nano bubble technique.[15] In basic knowledge of the system, gas bubbles produced into any liquid has the opportunity of producing discharged plasma.[26] When electrical current is released in micro/nano water bubbles in a short period of time, many reactive species such as radicals (hydroxyl radicals, $OH^{\bullet}$; superoxide radical, $O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), etc. are generated,[16,27,28] which was confirmed by optical emission spectroscopy (OES) in a previous study.[15] Therefore, those free radicals can oxidize any substance directly, especially the GSH in this study.[17] This was confirmed by results of percentage GSH loss with time response (5-20 min) (Figure 5.A). In addition, the preliminary study confirmed that free radicals in micro/nano water bubbles react directly with GSH without heated effects. Figure 7 shows that 60 and 80 degrees Celsius in a water bath involved less than 10% GSH, which was non-significant (92.32±1.4 % and 92.63±1.3%), when compared with room temperature. Whereas the GSH levels in HV micro/nano water bubbles at either 60 or 80 degrees Celsius were decreased significantly. The results showed extracts that contained GSH at 100 mg/mL in the micro/nano-bubble water after mixing, and before electrical discharge. They also showed scavenging activity, the same as positive antioxidant L-ascorbic acid in extract, and protected GSH with dose responses by direct scavenging of all free radicals in the system.

Finally, the activity of star fruit extract on the inhibition of TNF-α via inflammatory cells was studied as well, because a previous study found inflammatory activity of star fruit juice - sour type - by reducing the TNF-α level in elderly people.[9] Therefore, the study of star fruit - sweet type - was investigated in the LPS-activated macrophage cell line (J774A.1). The result showed that star fruit extract, as well as the standard anti-inflammatory agent, hydrocortisone, could reduce TNF-α with dose response at 62.5, 125, and 250 µg/mL. The possible mechanism of anti-inflammatory star fruit extract could be explained by its active and total phenolic compounds or total flavonoids. Previous evidence showed that phenolic-enriched extract of *S. glabra* (PEESG) could suppress TNF-α in LPS-induced RAW2647 cells,[29] with the same activity as Brazilian green propolis in J774A.1 macrophage cells.[30] However, this study cannot claim absolutely that star fruit extract - sweet type - has anti-inflammatory properties via its total phenolic or flavonoid compounds in vivo or humans, and this should be studied in the future.

Conclusion

This study summarizes that star fruit extract - sweet type - contains total phenolic and total flavonoid compounds as well as ascorbic acid. It can scavenge organic cation and free radicals, as well as NO in an *in vitro* model. Moreover, star fruit has anti-inflammatory activity in macrophage cells.

Acknowledgements

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Conflicts of interests

The authors report no conflicts of interests in this study.

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Biomechanical differences between sit-to-stand performances using one leg and two legs in young adults

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ABSTRACT

Background: Sit-to-stand (STS) test is widely used as a functional test for the assessment of lower extremity function in the elderly. Performing the STS movement with one-leg was introduced as an assessment of lower extremity muscle strength in young adults; however, the biomechanical differences between the traditional two-leg STS movement and one-leg STS movement have not been reported. The purposes of this study were to characterize and compare the kinematic and kinetic differences between the one-leg and two-leg STS movements.

Materials and methods: Fifteen young adults (8 men and 7 women) with mean age 26.18±3.88 years participated in this study. The kinematic and kinetic data during one-leg and two-leg STS testing conditions were collected and analyzed using force plates and a three-dimensional motion analysis system.

Results: Performance time was significantly longer in the one-leg STS condition than the two-leg STS condition (p<0.001). The peak joint angular positions of the hip, knee, and ankle were not different between the two STS testing conditions. All kinetic variables of the one-leg STS condition were significantly higher than those of the two-leg STS condition (p<0.05), except peak knee joint power in the concentric phase.

Conclusion: The more demanding task of the one-leg STS condition led to several changes in the joint moment and joint power of the lower extremity. The hip extensor and ankle dorsiflexor muscles demonstrated significant roles in addition to the knee extensor muscles during the one-leg STS task.

Introduction

Sit-to-stand (STS) test is often used as a functional test of lower extremity (LE) muscle strength.[1-4] The traditional form of STS test uses both legs to perform the STS task. Performance time of STS tests was reported to have a significant correlation with strength of major lower limb muscles in healthy older community-living adults.[1,2] Due to relatively high LE muscle strength of young adults compared to older adults[5], several tests that require greater demand of the LE muscles have been proposed as a functional test for assessment of LE strength in young adults. A one-leg-rising test was formerly used to assess leg extensor muscle function in patients with hip and knee arthritis[6] and later was modified as a LE functional performance test in young soccer players.[7]

Recently, an alternate form of STS test was introduced to assess LE muscle strength in young adults called "one-leg STS test".[8] A one-leg STS test is defined as a test to measure the ability to perform repeated sitting to standing movement using one leg. Concurrent validity of a one-leg STS test was reported with significant moderate relationships between the strength of LE muscles and performance time of a five-repetition one-leg-STS test. The advantages of a one-leg STS test include ease of administration and suitability in

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clinical settings. Performance of STS results in mechanical changes from a stable position to a less stable position with a higher body's center of mass position and a smaller base of support. Therefore, it is a challenging movement with great biomechanical demands, requiring joint torque as well as precise control of the body's center of mass within the base of support to complete the task.[9,10]

Biomechanical analyses of traditional two-leg STS movement have been extensively reported.[11-13] On the other hand, there is a paucity of research examining the biomechanical measures of a one-leg STS task. With greater demand placed on the LE muscles, individuals may exhibit different motion strategy and distributions of the hip, knee, and ankle joint moments when performing the sit-to-stand task with only one leg. Comparison of the mechanical differences between performance of the one-leg STS and the traditional two-leg STS tests is needed in order to provide basic information of this alternate form of STS test. Findings of the present study may aid the therapists for appropriate selection of the type of STS test for their clients in different age groups. Therefore, this present study aimed to investigate the kinematic and kinetic variables of a one-leg STS movement in healthy young adults and compare with those of the two-leg STS movement

Materials and methods

Fifteen young, healthy adults (8 men and 7 women; mean age 26.18±3.88 years; mean mass 55.05±11.09 kg; mean height 1.65±0.97 m.) participated in the study. The sample size was calculated by the G*Power 3.1.7 program for t-tests: Mean difference between two dependent means (matched pairs). To achieve 80% statistical power, effect size of 0.7 (based on a previous study comparing trunk kinematics between the one-leg and two-leg STS movements[14]) with an alpha level of 0.05, fifteen participants were required. Participants were included in the study if they were between the age of 20 and 40 years and excluded if they had neurological or musculoskeletal disorders that would affect the ability to perform STS movements. The study protocol was approved by the institutional review board of Mahidol University (MUICRB, COA no. 2016/180.2810). All participants gave written informed consent before the data collection process.

Kinematic and kinetic data were collected using the Vicon™ Motion Analysis System (Vicon™ Motion Systems Ltd, Oxford, UK), consisting of ten cameras with a sampling frequency of 100 Hz, integrated with two force platforms (AMTI OR6-7 Series 4000, Advanced Mechanical Technologies Inc., Boston, USA) with a sampling frequency of 1000 Hz. Thirty-four reflective markers were placed on the participant's body according to the Plug-In Gait-Full Body standards available within the Vicon Motion system. The 3-D motion and force data from the selected trials were processed using Vicon Nexus software (version 3.5.1) and were filtered with a 4th-order Butterworth zero-lag filter, with a cut-off frequency of 8 and 20 Hz, respectively. The kinematic and kinetic variables were calculated using the Vicon Plug-in Gait Model.[15]

Each participant performed both one-leg and two-leg STS testing conditions (Figure 1). A sit-to-stand test with five repetitions was used in order to compare to a common form of standard two-leg STS test[2] (a five-repetition chair stand test). Half of the participants performed the one-leg STS testing condition first while the other half performed the two-leg STS testing condition first. An armless, height-adjustable chair was used in the testing. All trials were performed with bare feet. Participants began each trial in a seated position with their arms folded across their chests and their feet shoulder-width apart and placed slightly behind the knee joint. The seat height was adjusted to the knee joint level such that the knee of the tested leg was set at 100 degree flexion. The verbal instructions were "Please stand up and sit down five times as quickly and safely as possible. Stand up until your legs are fully straightened and your buttocks are against the seat when you sit down, Ready and Start." Timing began on the command of the examiner and stopped when the participant's buttocks touched the seat after the fifth stand. Before beginning actual data collection, participants performed two practice trials to familiarize themselves with the test while the examiners made sure the motion capture and force plates functioned properly. Each participant performed two trials in each condition and the fastest of the two trials was used for data analysis. A three-minute rest was allowed between trials to avoid fatigue. Testing procedures of the one-leg STS testing condition were similar to the two-leg STS testing condition except using only the dominant leg to perform the STS task. The non-test leg (non-dominant side) was lifted just above the floor throughout the test and not allowed to assist the STS movement. The dominant leg was determined by leg dominant test.[16] Twelve participants out of 15 had right-leg dominance. Trials were discarded if the participant's non-tested foot touched the floor during the trial.

Figure 1 *Illustration of the sit-to-stand testing conditions a) one-leg sit-to-stand condition b) two-leg sit-to-stand condition.*

Kinematic variables included the peak joint angular position of the hip, knee, and ankle. The kinetic variables included the peak vertical ground reaction force (VGRF), peak joint moment and joint power of the hip, knee and ankle. Since the nature of the STS movement mainly occurs in the sagittal plane, only a sagittal plane evaluation of the variables was of interest in this study. VGRF data and the hip angular position were used to identify the event and phase of the STS test. Each of the sit-to-stand task comprised of five repetitions (Figure 2). The data from the second to fourth repetitions were used for data analysis. Each repetition was divided into the sit-to-stand part (concentric phase) and stand-to-sit part (eccentric phase). Vertical lines in Figure 2 were added to demonstrate the separation of the two parts. The joint angular positions and kinetic variables of each repetition were time-normalized to create ensemble-averaged across participants to assist visual inspection. The mean difference of the kinematic and kinetic variables between STS test conditions was calculated by subtracting the value of the two-leg STS test from that of the one-leg STS test. The percent mean difference is the proportion of the mean difference divided by the average of the two values.

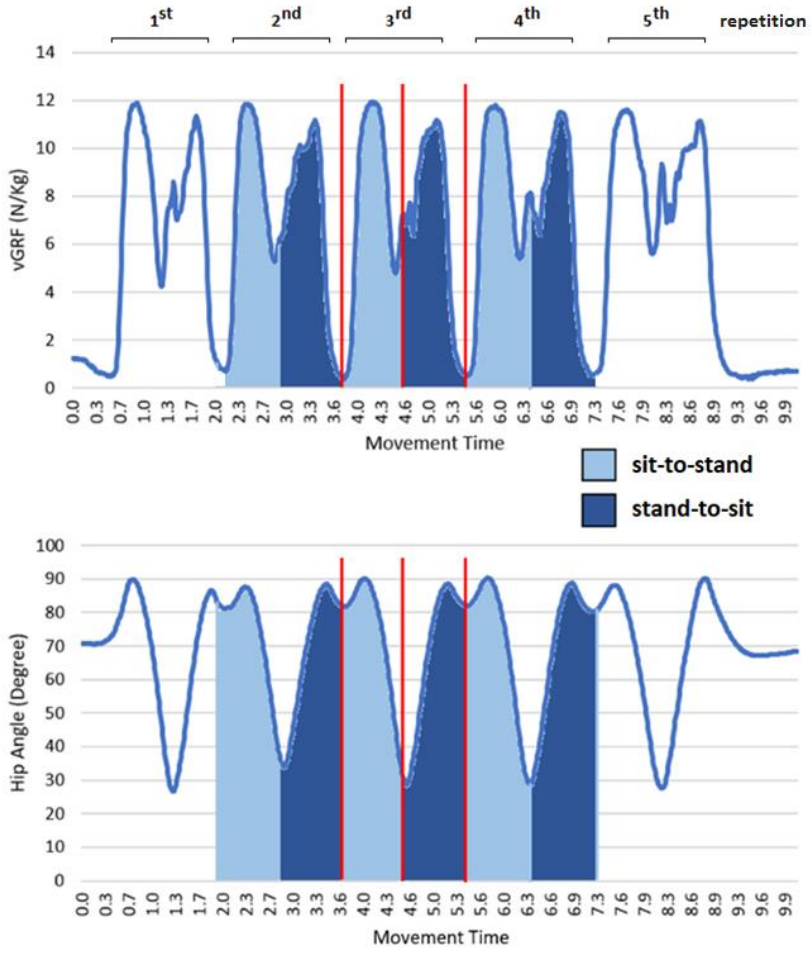

Figure 2. *Typical VGRF and hip joint angle profiles of a sit-to-stand test.*

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics (version 23) for Windows. The Kolmogorov-Smirnov test was used to assess the normal distribution of the data. Paired t-test was used to compare the differences in the joint angular displacement and kinetic variables between the one-leg and two-leg STS tests. Statistical significance level was set as $p<0.05$ for all analyses.

Results

Mean performance time of the one-leg STS condition was significantly longer than that of the two-leg STS condition ($p<0.001$). The mean joint angular positions of the hip, knee, and ankle were not different between the two STS testing conditions. The means and SDs of the performance time and peak joint angular positions of both STS conditions are summarized in Table 1.

Table 1 Comparison of the performance time and joint angular position between the one-leg and two-leg STS testing conditions

Variables	One-leg STS	Two-leg STS	Mean difference	% Mean difference	*p* value
Performance time (s)	11.63±2.96	8.27±1.42	3.36	33.77	<0.001**
Peak joint angular position (deg)					
Max hip angle	81.99±8.43	83.62±6.85	-1.63	-1.97	0.514
Min hip angle	8.22±9.14	6.67±8.65	1.55	20.82	0.142
Max knee angle	86.35±5.67	87.12±4.79	-0.77	-0.88	0.445
Min knee angle	7.14±9.12	6.42±6.12	0.72	10.62	0.216
Max ankle angle	21.91±5.79	20.33±3.42	1.58	7.48	0.416
Min ankle angle	3.19±4.16	2.90±3.75	0.29	9.52	0.614

Note: *** significantly different at p<0.01*

VGRF, joint moment and joint power of the one-leg and two-leg STS tests were generally similar in profile pattern but different in magnitude. Illustrations of the ensemble -averaged data of the VGRF, joint moments, and joint powers are shown in Figures 3, 4 and 5, respectively. VGRF profile contains two separated peaks. The first peak occurs in the sit-to-stand portion (concentric phase) and the second peak occurs in the stand-to-sit portion (eccentric phase). The values of the peak VGRF, peak joint moment and peak joint power are shown in Table 2. All kinetic variables of the one-leg STS condition were significantly higher than those of the two-leg STS condition (p<0.05), except peak knee joint power in the concentric phase.

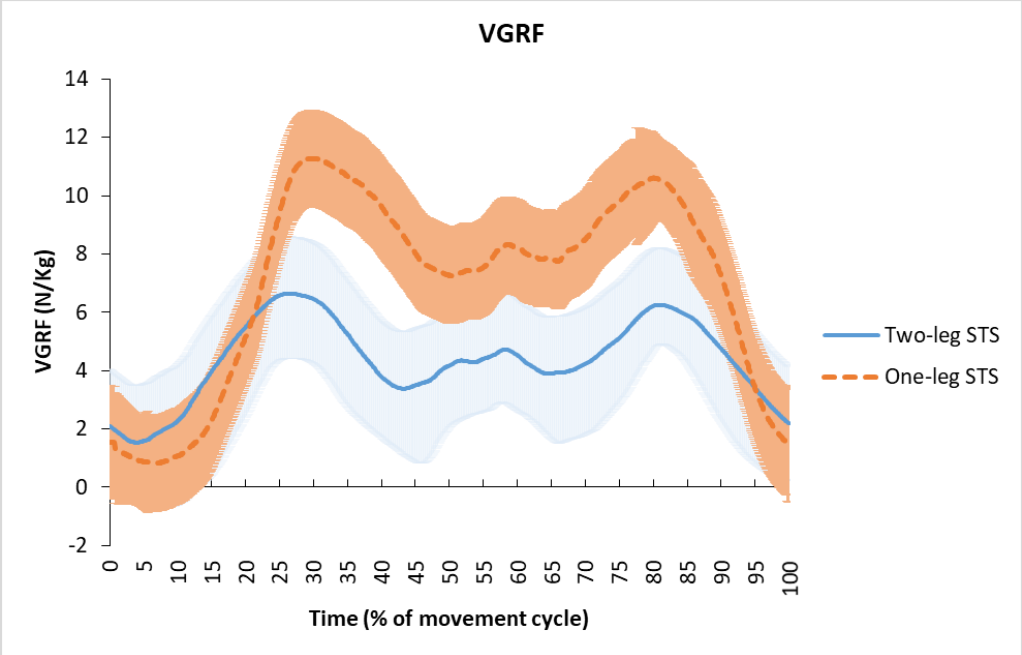

Figure 3. *Ensemble-averaged data of the vertical ground reaction force (VGRF).*

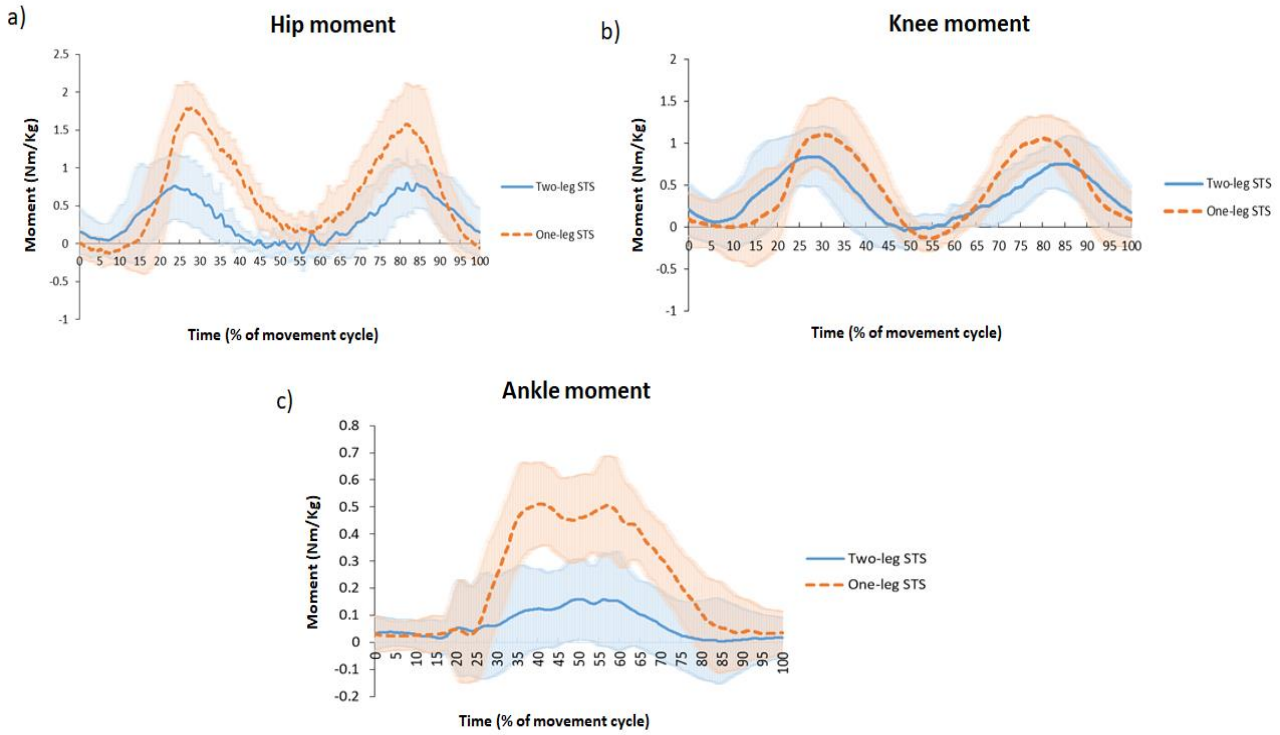

Figure 4. *Ensemble-averaged data of a) hip joint moment, b) knee joint moment and c) ankle joint moment.*

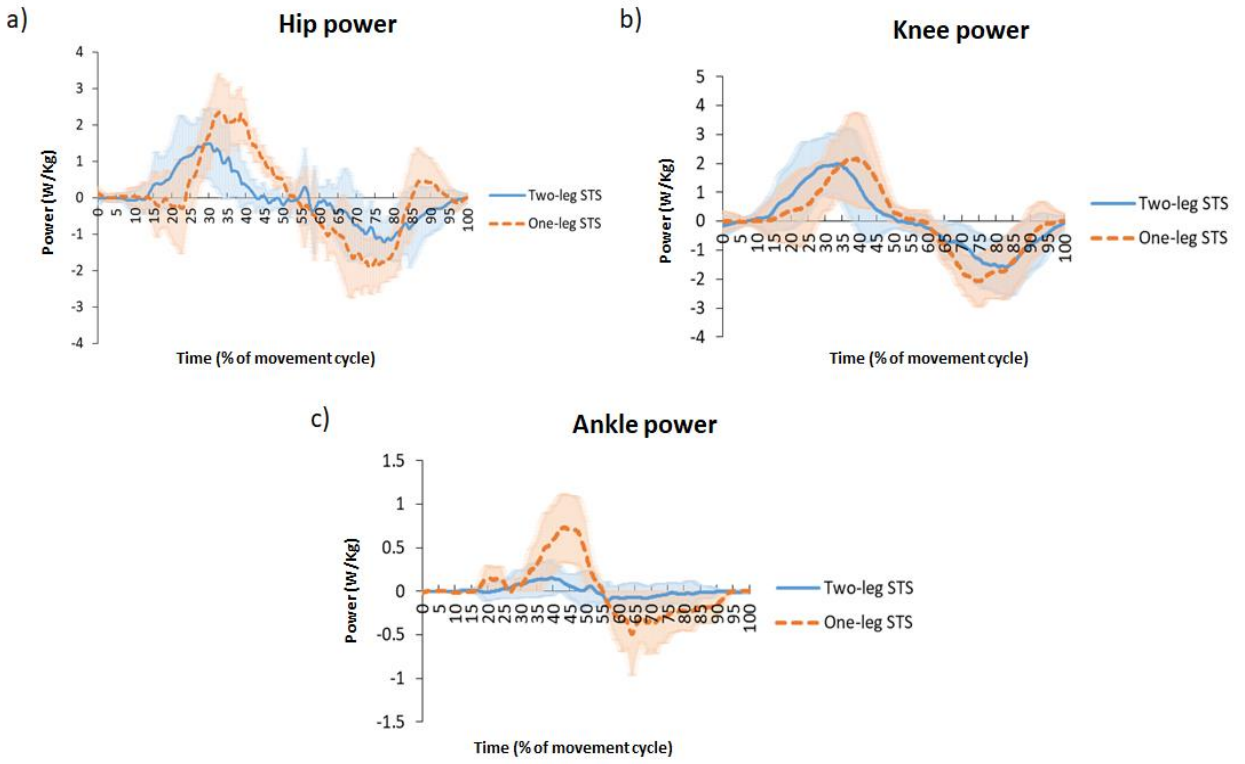

Figure 5. *Ensemble-averaged data of a) hip joint power, b) knee joint power c) ankle joint power.*

Table 2 Comparison of the kinetic variables between the one-leg and two-leg STS testing conditions.

Kinetic variables	One-leg STS	Two-leg STS	Mean difference	% Mean difference	*p* value
Peak VGRF (N/kg)					
Concentric phase	11.61±1.07	7.38±0.92	4.23	44.55	<0.001**
Eccentric phase	11.32±1.05	6.70±0.94	4.62	51.28	<0.001**
Peak joint moment (Nm/kg)					
Hip - concentric phase	2.03±0.26	1.02±0.33	1.01	66.23	<0.001**
Hip - eccentric phase	1.92±0.40	1.00±0.39	0.92	63.01	<0.001**
Knee - concentric phase	1.35±0.30	1.13±0.24	0.22	17.74	<0.001**
Knee - eccentric phase	1.27±0.28	0.92±0.21	0.35	31.96	<0.001**
Ankle - concentric phase	0.73±0.21	0.15±0.07	0.58	131.82	<0.001**
Ankle - eccentric phase	0.71±0.16	0.22±0.10	0.49	105.38	<0.001**
Peak joint power (Watt/kg)					
Hip - concentric phase	3.22±0.72	2.25±0.99	0.97	35.47	0.003**
Hip - eccentric phase	3.00±0.78	2.17±0.83	0.83	32.11	<0.001**
Knee - concentric phase	3.01±0.91	2.90±1.10	0.11	3.72	0.625
Knee - eccentric phase	2.42±0.70	2.12±0.72	0.30	13.22	0.024*
Ankle - concentric phase	0.57±0.14	0.24±0.15	0.33	81.48	<0.001**
Ankle - eccentric phase	0.58±0.44	0.21±0.11	0.37	93.67	0.002**

***Note**: * significantly different at p < 0.05, ** significantly different at p<0.01*

Discussion

The results revealed that several biomechanical differences exist between the two STS testing conditions. Participant's body weight is considered an external load that the leg muscles have to overcome during standing up and sitting down. For a usual STS task using two legs, the external load is opposed by muscles of both legs, whereas in the one-leg condition, this same external load is placed solely on one leg which induced a strategy change in STS performance. It took 3.36 seconds longer for the participants to complete the one-leg STS condition compared with the two-leg STS condition. The results are in accordance with Savelberg el al[17] who examined the effect of load added to the body while performing a traditional two-leg sit-to-stand task. Increased extra load from 30% to 45% of body weight resulted in increased movement time, increased maximum joint moments at hip, knee and ankle joints and changes in muscle activation patterns of major leg muscles. In this study, the kinematic variables (joint angular positions) were not different between the two testing conditions. The LE joint position of the tested leg at the starting position was the same for both STS testing conditions. For each repetition of the STS tests, the participants returned to sit at the same seat height and stood up to full upright position. Therefore, the ranges of motion of the hip, knee, and ankle joints were not different between STS conditions.

Almost all kinetic variables were found to be different between the two STS testing conditions. Increased VGRF indicated larger net muscle force is generated by the acting leg muscles during the one-leg STS condition.[18] VGRF of the one-leg STS condition increased by 4.23 N/kg (44.55%) and 4.62 N/kg (51.28%) in the concentric and eccentric phases, respectively. Our results are supported by previous studies investigating the effect of increasing load on ground reaction force during squatting which is a similar movement to STS mainly using the LE muscles. Kellis el al[19] examined the effect of increasing load on the ground reaction force during barbell squat and found that GRF increased significantly as external load increased. Dali et al[20] found that deep squatting generated the highest VGRF compared to semi and half squatting.

It is clear that major leg muscles were more activated to control the whole body up and down repeatedly throughout the one-leg STS condition. Previous studies reported that the knee and hip extensors play a major role in the sit-to-stand movement.[21-23] In this study, although all the hip, knee, and ankle extensor moments and joint power significantly increased during the one-leg STS condition, it is interesting that the largest increase in joint moment and power occurred at the ankle joint. The mean increases of the ankle joint moment were over 131 and 105 percent in the concentric and eccentric phases, respectively, indicating the crucial role of the ankle muscles in stabilizing the foot and lower leg in order to achieve sufficient balance during this demanding task[24].

For the two-leg STS condition, the largest joint moment was originated from the knee joint. However, the higher moment about the knee during the two-leg STS condition

is shifted to proportionally higher moments about the hip and ankle during the one-leg STS condition. This could be due to the more demanding task of the one-leg STS condition which causes this change in the net moment. The hip extensor muscles which have larger muscle size were recruited more to produce sufficiently net joint moment to perform the task. The peak hip extensor moment increased over 60 percent revealing the synergistic role of the hip extensor muscles during the one-leg STS condition. Savelberg et al[17] explained that the primary adaptation in response to added load is decreasing in movement time and increasing in knee extension moment. If the maximum capacity of the knee extensor strength is sufficient, individuals can perform the task without inducing a strategy change. Secondly, if a strategy change has been induced, the hip extension torque is more required. The latter explanation is in line with our results which found that the hip extensor muscles moment increased with the one-leg STS condition indicating that the hip strategy is preferred as the one-leg STS task required greater control of dynamic balance.

The results of the study indicated that compared to the traditional two-leg STS test, the one-leg STS test is a challenging task which is suitable for assessment of LE muscle function in young adults as it demands greater amount of force production from the LE extremity muscles to complete the STS task. However, this present study had some limitations. First, we investigated only the one-leg STS movement performed by the dominant leg. It might be possible that person may perform differently on their non-diminant side. However, Steingrebe el at[25] reported no significant differences in knee joint loading between the dominant and the non-dominant side during a unilateral sit-to-stand movement. Second, direct measurement of the LE muscle strength was not done in this study. Therefore, we cannot directly explained how much of the maximum strength capacity of the LE muscles would be required for the one-leg STS movement compared to the typical two-leg STS movement. All the limitation issues should be further investigated in future study.

Conclusion

Compared to a typical two-leg sit-to-stand movement, there was an increase in performance time of a one-leg sit-to-stand test. The patterns of angular displacements of the hip, knee and ankle joints of the two STS sit-to-stand movements were generally similar. In addition, the more demanding task of the one-leg STS condition led to several changes in the joint moment and joint power of the lower extremity. The hip extensor and ankle dorsiflexor muscles demonstrated significant roles in addition to the knee extensor muscles during the one-leg STS task.

Conflicts of interests

The authors declare no conflicts of interests.

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Performance of the Determine HIV-1/2 Combo rapid test for detection of acute/early HIV-1 infection

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ABSTRACT

Background: New guideline from CDC for HIV testing recommended the 4th generation assay as the primary screening test to avoid late diagnosis and to shorten the window period. The first point-of-care HIV assay named Determine HIV Combo was used in high prevalence setting. The capacity of this assay detects HIV infection more rapid than IgM/IgG laboratory-based assays. Sensitivity of HIV-1 p24 antigen (Ag) detection was lower than those of laboratory-based 4th generation assays. However, the capacity to detect acute/early HIV-1 infection in high risk subjects is needed to evaluate.

Objectives: To evaluate the performance of Determine HIV Combo in seroconversion plasma specimens of acute/early HIV-1 patient.

Materials and methods: Twelve seroconversion plasma specimens from 5 acute/early patients were diagnosed by Elecsys HIV Ag, 4th generation ECLIA (Elecsys HIV Combi PT) and supplemented by NAAT (viral load) to evaluate the performance of Determine HIV Combo sensitivity. McNemar's exact test was applied to compare the difference in reactivity during acute/early infection between tests. Specificity was evaluated with 96 HIV-1 negative plasma specimens.

Results: Seroconversion sensitivity of Determine HIV Combo was 91.67% (11/12, p=0.32) compared to 4th generation ECLIA and Elecsys HIV Ag results. There were 3 discordant results with 3rd generation HIV POCT (p=0.14). The reactivity of HIV p24 Ag detection which compared to Elecsys HIV Ag, were 41.67% (5/12, p=0.0021). Determine HIV Combo had 8.33% (1/12) false negative result and 100% specificity. The first positive HIV p24 Ag and antibody results from Determine HIV Combo were found on 14 days and 21 days, respectively.

Conclusion: Determine HIV Combo provides poorer antigen sensitivity compared to HIV Ag and viral load performance, p24 Ag. However, Determine HIV Combo can detect acute/early HIV-1 infection at more than 13 days after sexual exposure. It can improve HIV rapid test to decrease the window period, expand access to HIV testing and prevent HIV transmission.

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Introduction

The 4th generation or Combo HIV-1/2 antigen/antibody (Ag/Ab) immunoassays was used as the primary screening test in the algorithm recommended by the Centers for Disease Control[1] and combination assays to detect HIV-1 p24 antigen has affected to reduce diagnostic window period and improve detection of HIV-1 infections when compare to the 3rd generation (Ab only) assays.[2-4] Early diagnosis and early treatment of acute HIV infection by HIV combo tests were accepted to diagnose HIV-infected individuals for early antiretroviral treatment, which can reduce transmission, improve quality of life and health outcomes for infected persons and their partners.[5] USA FDA approved seven HIV Ag/Ab assays for use in the U.S. and one of those is Determine HIV Combo rapid test which using fingerstick blood, venous whole blood, serum and plasma. The results can be read in 20 minutes. It was commonly used in non-laboratory settings and can differentiate HIV-1 p24 Ag from HIV-1/2 Ab reactivity. Recently, Thailand-FDA has approved another new Alere HIV Combo assay in August, 2018.[6] Increasing early HIV diagnosis and connecting to care in HIV care units is a strategy to reduce HIV transmission and target the next set of goals to end AIDS by 2030 (95-95-95 for treatment: 95% of people living with HIV knowing their HIV status; 95% of people who know their status on treatment; and 95% of people on treatment with suppressed viral loads). However, the potential of Determine HIV Combo for detection of acute/early HIV infection is needed for further evaluation.

Materials and methods

Study designs

HIV-1 specimens

Plasma HIV diagnosis testing in high risk population (men who have sex with men: MSM, transgender: TG and Female sex worker: FSW) was performed. The samples were recruited from Reach-Recruit-Test-Treat- Retain (RRTTR) program, AIDS ZERO Plan of Thailand at District drop in center (DIC) and three mobile HIV-testing services including Bangkok, Chon Buri, and Phuket Province during January-June 2015. All participants were asked for given writing or verbal consent received counseling before HIV testing. The study was approved by the Department of Diseases Control, Ministry of Public Health, Thailand. Among 529 participants, five acute/early HIV-1 infection was characterized including four MSM and one FSW. Twelve serial EDTA plasma specimens with HIV Ag positive were studied by Elecsys HIV Ag which were used to evaluate the performance of Determine HIV Combo assay for its seroconversion sensitivity. Specificity of this assay was compared to 96 HIV-1 negative plasma specimens tested by Elecsys HIV Combi PT (4th generation assay).

HIV assays

The 4th generation rapid test, Determine HIV Combo assay (Determine HIV-1/2 Ag/Ab Combo, Alere Inc., Japan) was tested in 12 acute/early HIV-1 seroconversion plasma specimen to compare sensitivity with 4th generation assay (Elecsys HIV Combi PT, Roche Diagnostics GMBH, Germany) and HIV Ag assay (Elecsys HIV Ag, Roche diagnostics, GMBH, Germany). Days after sexual exposure and temporal trend of laboratory tests were compared with 3rd generation HIV POCTs, including Alere Determine HIV -1/2 (Inverness Medical Japan Co., Ltd., Japan), DoubleCheckGold Ultra HIV1&2 (Organics Ltd., Israel) and SD Bioline HIV-1/2 (Standard Diagnostics, Inc. Korea). Nucleic Acid Amplification Test (NAAT) viral load (Cobas AmpliPrep/ CobasTagMan HIV-1 test, version 2.0, Roche Molecular Systems, Inc., USA) was used to confirm acute/early infection at the first visit. All tests were performed by the protocol recommended by manufacturers.

Analysis of assay performance in acute/early HIV-1 infection

Seroconversion sensitivity of Determine HIV Combo assay was calculated from the positive test results versus the days before the 4th generation assay (Elecsys HIV Combi PT), HIV Ag assay (Elecsys HIV Ag) and/or NAAT viral load test (Cobas AmpliPrep/ CobasTagMan HIV-1 test) became positive in 12 plasma specimens of seroconverts. McNemar's exact test was used to compare the difference in reactivity during acute/early infection between tests. Specificity was calculated by the results of 96 HIV-1 negative specimens.

Results

Reactivity of the Determine HIV Combo was analyzed and compared to 4th generation assays in 12 seroconversion specimens. The sensitivity of Determine HIV Combo was 91.67% (11/12), compared to 4th generation ECLIA, Elecsys HIV Combi PT and Elecsys HIV Ag (p=0.32). Of the 12 seroconversion acute/early HIV-1 specimens tested, Determine HIV Combo gave 41.67% (5/12) reactive for p24 Ag (Ag+/Ab+, Ag+/Ab-), whereas 25% (3/12) were reactive for p24 Ag only. Of 66.67% (8/12) were found HIV Ab band, and 6 of them (50%) were reactive for antibody only. There was one non-reactive in both Ag and Ab by Determine HIV Combo. There were 3 specimens showing discordant results with 3rd generation HIV POCT. The HIV p24 Ag reactivity of Determine HIV Combo compared to Elecsys HIV Ag, was 41.67% (5/12, p=0.0021). The specificity of Determine HIV Combo compared with 4th generation ECLIA, Elecsys HIV Combi PT was 100% (Fig. 1). Determine HIV Combo detected the first Ag p24 and antibody positive results after sexual exposure at 14 days and 21 days, respectively (Table 1). HIV viral load of all participants at the first visit were 117,800 - >10,000,000 copies/ml.

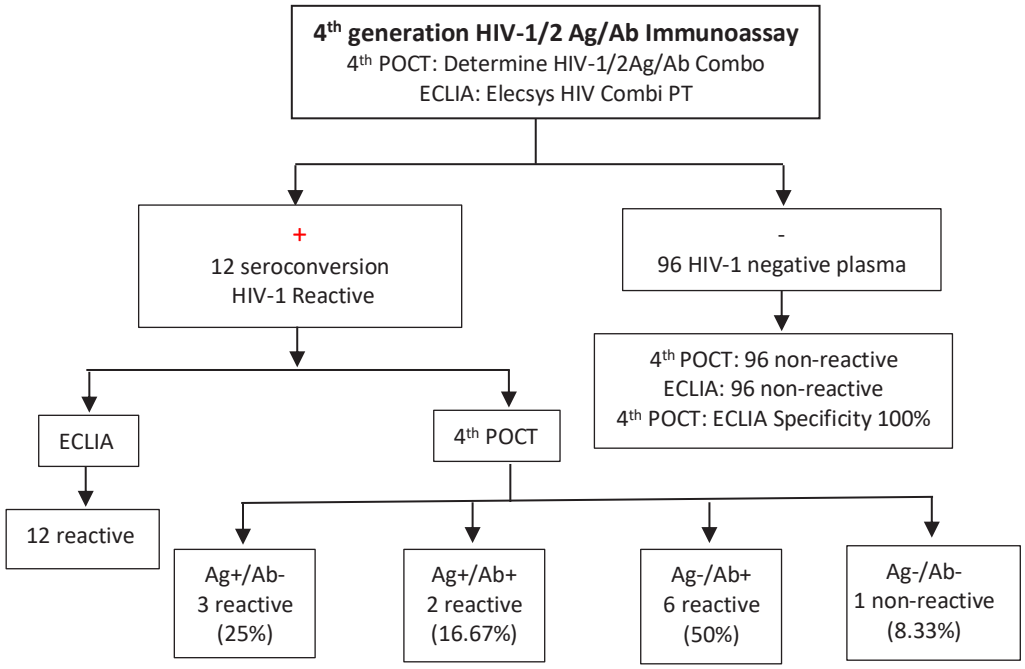

Figure 1. *Laboratory HIV testing algorithm for seroconversion plasma specimens in this study. POCT: Point of care Testing, ECLIA; Electrochemiluminescence Immunoassay, Ag: antigen, Ab: antibody.*

Table 1 Days after exposure and temporal trend of HIV testing in 12 seroconversion specimens.

No.	Risk group	Days after sexual exposure	4th generation			3rd generation POCT			Elecsys HIV Ag (S/CO) Positive>0.9	HIV RNA (copies/ml)
			Determine HIV Combo		Elecsys HIV Combi PT (S/CO) Positive>1	Alere Determine HIV-1/2 Ab	Double CheckGold Ultra HIV1&2	SD Bioline HIV-1/2		
			Ag	Ab						
1	MSM	14	+	-	+(111.3)	-	-	-	+* wp (4.9)	>10,000,000
2		19	+(pale)	-	+(338.4)	-	-	-	+(11.9)	Not done
3		21	-	+(pale)	+(443.2)	+(pale)	+(pale)	+(Pale)	+ (8.3)	Not done
4	MSM	22	+	+	+(504.4)	+	+(pale)	+(pale)	+*(8.7)	474,000
5		32	-	+	+(646.8)	+	+	+	+(11.1)	Not done
6	MSM	22	-	+	+(431.2)	+	+	+	+*(9.6)	291,000
7		27	-	+	+(332.2)	+	+	+	+wp(5.8)	Not done
8		35	-	+	+(713.8)	+	+	+	+wp(3.8)	Not done
9	MSM	25	+	+	+(89.4)	+ (pale)	+ (pale)	+(pale)	+*(7.3)	380,000
10		33	-	+	+(423.0)	+	+	+	+wp(5.4)	Not done
11	FSW	13	-	-	+(59.9)	-	-	-	+*(5.7)	117,800
12		16	+(pale)	-	+(89.8)	-	-	-	+(9.1)	7,480,000

+ (pale): reactive with pale color of band, wp= weakly positive, + : Elecsys HIV Ag positive (specimen no.1,4,6,9 and 11) confirmed HIV Ag positive by HIV Ag neutralization test.*

Discussion

Determine HIV combo is 4th generation rapid test device capable of determining 26 HIV-1 p24 antigen and both of IgM & IgG HIV-1/2 antibodies in serum, plasma and whole blood. Sensitivity and specificity of Determine HIV Combo according to this study were 91.67% and 100%, respectively compared to 4th generation ECLIA, Elecsys HIV Combi PT. The sensitivity shown in package insert are 99.9% and 100%, using all types of specimens for low risk subjects and ranges from 98.9% (serum) to 99.7% (whole blood) for high risk subjects.[7] Previously report and our finding indicated that antibody sensitivity is comparable to package insert for high risk subjects, but the antigen component is not detected in most acute/early HIV-1 infections revealed by laboratory 4th generation assays.[8-9] Like in our cases, performance of Determine HIV Combo was evaluated on seroconversion plasma specimens from patients with laboratory algorithm defined acute/early HIV-1 infection. Determine HIV Combo failed to detect early HIV-1 infection in 8.33% (1/12). The studies of Faraoni and Cohen also showed that Determine HIV Combo (detecting both IgM & IgG antibodies and p24 antigen) had been found to be less accurate in identifying the acute phase of HIV infection.[10-11] Result from the present study evidenced that time to reactivity for Determine HIV Combo is 7 days earlier than 3rd generation assays (POCT) in one MSM subject (Table 1). This was the same as studies of CDC that compared all US FDA-approved tests in the same plasma specimens collected from individuals during seroconversion. It showed that Determine HIV Combo can detect HIV infection 1-2 weeks before other 3rd generation rapid tests, and 3-4 days after 4th generation assays.[12] The previous data from Delaney and colleagues studied on plasma specimens showed that the median time to reactivity of Determine HIV Combo is 2.3-3.5 days faster than laboratory-based 3rd generation assays.[13] We found 3 plasma specimens which were detected by Determine HIV Combo, meanwhile 3rd generation assays (POCT) could not detect. There were 2 cases of acute HIV-1 infections which had high viral load and HIV Ag positive. One (MSM) of two subjects had viral load >10,000,000 copies/ml and a positive Determine HIV Combo result at 14 days after sexual exposure. In contrast, the other (FSW) failed to diagnosis with Determine HIV Combo and had HIV viral load 117,800 copies/mL at 13 days after sexual exposure. While 3 days later, HIV viral load reached to 7,480,000 copies/mL and Determine HIV Combo was positive Ag band (Table 1). This revealed that early diagnosis of HIV infected cases and early treatment can decrease the spread of HIV to their partners. Our finding revealed performance of the Determine HIV Combo test can be detected the infection 14 days after sexual exposure, which is 7 days faster than 3rd generation POCT. (Table 1) Thus, active HIV case finding in high risk, hard to reach populations, such as MSM and FSW, need non-laboratory based HIV testing in field, non-instrument based and rapid turnaround time.[14] The 4th generation POCT assay seems to be appropriated for the first HIV screening test after sexual exposure more than 13 days. Therefore, when less than 14 days, when reporting negative results by Determine HIV Combo, high risk population who have sexual risk behavior should be performed supplement tests such as HIV Ag assay, laboratory-based 4th generation HIV tests and/or HIV RNA assay because they can migrate and spread transmission to others.

Conflicts of interests

No conflict of interest

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Prevalence and factors associated with chronic ankle instability among children aged 7 to 12 years

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ABSTRACT

Background: Ankle sprain is the most common cause of chronic ankle instability (CAI). After a sprain, CAI and related components may develop and adversely affect movement performances. Prevalence of CAI in specific groups of children has been reported, however, the prevalence in typical children is lacking.

Objectives: This study aimed to determine the prevalence of CAI and related components in school-age children. Additionally, factors associated with the components were also examined.

Materials and methods: Three hundred and eighty-eight children aged between 7 and 12 years with no past or present serious diseases or disabilities were recruited from normal schools. They were interviewed and assessed to identify CAI components and risk factors. Children with at least one of the 3 components of CAI, including perceived instability (PI), mechanical instability (MI) and recurrent sprain (RS) were recorded as having CAI.

Results: The children's mean age was 9.76±1.55 years, and 57% of them were girls. There were 142 children with at least one component of the CAI. Therefore, the prevalence of CAI among the population was 36.60 %. The prevalence of MI, PI and RS were 11.6%, 35.3% and 27.3%, respectively. Significant variables on bivariate analyses (p<0.05) for related components of CAI were overweight, sport participation, living in urban area, moderate degree of initial ankle sprain and poor standing balance in eyes closed condition. After adjusting for the significant variables, overweight (aOR: 1.083, [95%CI: 1.036-1.192], p<0.001) and poor standing balance in eyes closed condition (aOR: 1.142 [95% CI: 1.194-1.311], p<0.001), were associated with RS. Overweight (aOR: 1.229 [95%CI: 1.063-2.264], p<0.001), sport participation (aOR: 1.192 [95%CI: 1.052-3.308], p=0.013), moderate ankle sprain (aOR: 1.143 [95%CI: 1.038-3.541], p=0.004) and poor standing balance in eyes closed condition (aOR: 3.476 [95% CI: 1.872- 6.453], p=0.006) were associated with PI. Moderate ankle sprain (aOR: 1.099 [95%CI: 1.027-4.370], p<0.001), and poor standing balance in eyes closed condition (aOR: 4.251 [95% CI: 1.248- 14.485] p=0.021) were associated with MI.

Conclusion: CAI and related components were existed among typical school-age children. The risk of all CAI components was high in children with poor standing balance. Overweight children were at higher risk of RS and PI. Children with moderate degree of ankle sprain were at higher risk for developing PI and MI. Further studies are recommended to develop preventive managements for this population.

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Introduction

Ankle is the most commonly injured joint of the body because it has to support and distribute body weight through the foot while performing weight-bearing activities.[1-3] Ankle injury involves tendon and ligaments around the joints. It leads to instability of the ankle and can disturb proprioception and balance abilities.[4] After an ankle injury, chronic ankle instability (CAI) may develop and lead to several long-term residual signs and symptoms.[5] The term CAI was used to classify people with a history of at least an ankle sprain and present residual symptoms such as the sensation of ankle instability for at least 1 year post injury.[5] The CAI can be explained using two instability types, including mechanical and functional instabilities.[6] The criteria to describe CAI are mechanical instability (MI), functional instability (FI) or both. In 2016, the International Ankle Consortium's statement concluded that Hiller et al model can explain the inconsistencies in CAI research regarding to the misconception that CAI is a homogeneous condition.[7] This statement proposed that CAI should be considered as a heterogeneous condition including several homogeneous subgroups. Therefore, it was concluded that CAI consists of at least one of three related components including perceive instability (PI), mechanical instability (MI) and recurrent sprain (RS).[5] Further research indicated that these related components can adversely affect physical activities of the sufferers such as decreased ability to play sport[8], inability to walk long distances[8] and cessation of sporting and occupational activities[9-10].

Many studies focused on exploring effective interventions for adults with CAI with the main aim of helping them to return to their pre-injury levels of physical activities or sports. The effects of CAI will be more serious if it happens in children population. Since children have less developed patterns of motor and postural control but require high level of physical activities in their environmental context.[11] Additionally, previous studies indicated that ankle sprains frequently occur in childhood population.[1-3] A systematic review reported that children with previous ankle sprain show a high rate of recurrent ankle injuries and CAI.[12] The prevalence of CAI in specific children populations including dancers, soccer players, and those with previous ankle trauma, was equal to or higher than that of adult populations.[12] Among the specific populations reviewed, the prevalence of PI, MI and RS was 23-71%, 18-47% and 22%, respectively.[12] A survey research determined the prevalence of CAI as identified by a self-reporting questionnaire in high school athletes (age 15.9±1.2 years) and collegiate athletes (age 19.6±1.2 years), revealed that the prevalence of CAI in high school and collegiate athletes was 31.1 % and 18.7%, respectively.[13] Of all athletes surveyed, 66.8 % of the high school athletes and 65.2% of the collegiate athletes had at least an episode of previous ankle sprain.[13]

The literature reported different prevalence of CAI depending on types of the study population and methods in identifying the condition. Specific groups of children such as athletes are different from children with specific diseases therefore the prevalence of CAI in each specific group of children cannot be compared or even used as the prevalence for typical children. In contrast, if the prevalence in typical children is determined, it can be used as the reference data for other specific groups. Moreover, a systematic review of many research studies on CAI was conducted and confirmed that there is no "gold standard" to identify CAI.[14] Previous studies used self-reporting questionnaires and recent research developed self-assessment tools for clinicians and researchers to help objectively assess patients with CAI.[5, 13] However, these tools were developed for adults and reported as having difficulties to use in children population.[12]

To better understand CAI in children, it is needed to determine the prevalence of CAI among typical children population. In addition, children` s information and outcomes from easy-to-use tests that associate with the CAI components should be identified. Physical therapists, school teachers and parents can use these data to early detection of CAI and to establish preventive managements for children in whom CAI is prevalent. Therefore, the purposes of this study were to determine the prevalence of CAI and its related components and to examine factors associated with the components in typical school-age children

Materials and methods

Participants

Five normal schools were selected with convenient sampling from Bangkok, Nakhon Pathom and Lamphun Provinces. Upon obtaining the permission to collect data from each school, the teachers and students were explained about the study protocol and the criteria of the research participants. Non-athletic children aged 7 to 12 years with no past or present serious illnesses or disabilities and whose parents gave informed consent for them to participate in this study were included and measured of their weight and height. All children with BMI between 5th and 95th percentile were then scheduled to undergo an interview and full assessments from the research team (Figure 1). The study protocol was approved by the university institutional review board for the protection of human subjects.

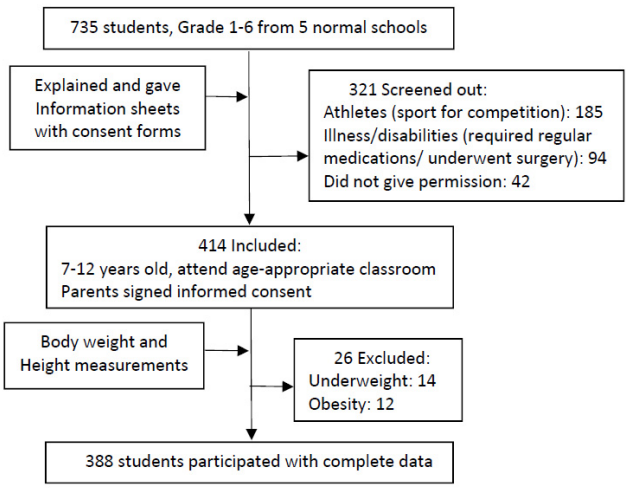

Figure 1. *Flow chart of participant recruitment*

Measurements

Children were interviewed to obtain information including age, gender, limb dominance, sport participation, living area, history of ankle sprain (severity of initial sprain, frequency of ankle sprain) and sensation of ankle instability. Talar tilt test, anterior drawer test, single leg stance eyes open (SLS EO) and eyes closed (SLS EC) conditions were then measured by a physical therapist with over 5 year experiences in pediatric physical therapy. Anterior drawer test and talar tilt test were measured using the standardized protocols.[15] Prior to the data collection, intra-tester reliability were examined and showed an acceptable level of reliability in performing anterior drawer test (ICC (3,1) = 0.75, p<0.05) and talar tilt test (ICC (3,1) = 0.82, p<0.05).

Data regarding to frequency of ankle sprain, sensation of ankle instability, talar tilt test and anterior drawer test were used to identify the presence or absence of the CAI components. The CAI components included perceived instability (PI), mechanical instability (MI) and recurrent sprain (RS).[5] Perceived Instability was self- report of having feeling of giving way or instability of the ankle. For the present study, the feelings that occurred within 1 year post initial ankle sprain were recorded. Mechanical instability was recorded if the talar tilt test or anterior drawer test were positive. Children who reported as having greater than one episode of ankle sprain were recorded as RS. Children with at least one of the 3 components of CAI were recorded as having CAI.

Data for examining the associations with the CAI components included gender, limb dominance, sport participation, living area, severity of the initial ankle sprain, body mass index (BMI), SLS-EO and SLS-EC. Score of "1" was recorded for female, left limb dominance, sport participation, living in urban area, moderate degree of the initial ankle sprain[16], overweight[17], positive SLS EO and positive SLS EC[18].

Body mass index was calculated and plotted against the age- and sex-specific BMI growth charts. The BMI-for-age status categories were used[17]. Normal weight was defined as BMI-for-age between the 5th and 85th percentiles, and the percentile of greater than 85th but below the 95th percentiles was defined as overweight [17].

Initial ankle sprain was graded on the basis of severity including mild, moderate and severe degrees.[16] For mild degree, children would report that the ankle was stable and they could walk with minimal pain immediately after the sprain. For moderate degree, children would report that the ankle was slight instability with moderate pain and they could not bear weight on the sprained ankle immediately after the sprain. For severe degree, walking was impossible because of the severe pain and swelling with marked instability of the ankle.

Single leg stance is commonly used to assess the ability of children to maintain static balance within a narrow base of support.[18] The children were instructed to stand on one leg as long as possible with a maximum of 30 seconds for each trial. They were bare foot and with hands on hips. The test was performed three times with eyes open (SLS EO) and three times with eyes closed (SLS EC). Before testing, they were allowed to practice once. A digital stopwatch was used to measure the standing duration. The measurement was completed when any of the followings occurred; 1) at 30 seconds, 2) any changes in position of the weight-bearing foot, 3) any body part except the weight-bearing foot touched the floor, and 4) open eyes during eyes closed condition. The durations of 3 trials were averaged to obtain the scores for SLS-EO and SLS-EC conditions. The average duration of less than 30 seconds was considered as poor standing balance in the condition.

Statistical analysis

Sample size was calculated using the expected proportion from a pilot study at 30%, and a margin of error of 0.05 with 95% confidence intervals.[19] The optimum sample size of 323 was required. The significance level was set at p<0.05. All statistics were analyzed using the statistical package SPSS version 19. Frequencies were calculated to determine the prevalence of CAI, PI, MI and RS.

A nonparametric chi-square test was used for association between CAI components and gender, BMI, limb dominance, sport participation, living area, severity of initial ankle sprain, SLS EO and SLS EC. In multivariate analysis, variables with p<0.05 in bivariate analyses were simultaneously analyzed by multiple logistic regression. Crude and adjusted odds ratios were computed.

Results

Demographic data

Three hundred and eighty eight children were recruited from five normal schools in Bangkok (32.7%), Nakhon Pathom (41.8%) and Lamphun (25.5%) Provinces. There were 167 (43%) boys and 221 (57%) girls. The average (± standard deviation) of the children's age and body mass index were 9.76 (±1.55) and 17.88 (±5.81), respectively. Of the 388 children, 270 (69.6%) reported previous history of ankle sprain(s). Table 1 shows distribution of previous history of ankle sprain among the population.

Table 1 Distribution of history of ankle sprain among the population (N=388).

Ankle sprain	Severity of initial ankle sprain	Recurrent sprain	n	%
No	-	-	118	30.4
Yes	Mild degree	No	121	31.2
Yes	Mild degree	Yes	69	17.8
Yes	Moderate degree	No	43	11.1
Yes	Moderate degree	Yes	37	9.5

Notes: *Severity of initial ankle sprain; Mild degree: ankle was stable and children could walk with minimal pain immediately after the sprain; Moderate degree: ankle was slight instability with moderate pain and children could not bear weight on the sprained ankle.*

Prevalence of CAI

Of the 388 children recruited, 142 (36.6%) had at least one component of the CAI. Therefore, the prevalence of CAI among the population was 36.6 %. The prevalence of MI, PI and RS were 11.6%, 35.3% and 27.3%, respectively (Table 2).

Table 2 Summary of prevalence of chronic ankle instability and related components (N=388).

Variables	n	%
Chronic Ankle Instability	142	36.6
Mechanical Instability	45	11.6
Perceived Instability	137	35.3
Recurrent Sprain	106	27.3

Factors associated with CAI components

In bivariate analysis, overweight, sport participation, living in urban area, moderate degree of initial ankle sprain and poor standing balance in eyes closed condition showed statistical associations with RS, PI and MI. Non significant variables on bivariate analysis (p>0.05) and being excluded from the multivariate analyses were gender, limb dominance and single leg stance with eyes open (SLS-EO) condition. Tables 3, 4 and 5 present results on the logistic regression analysis of factors associated with RS, PI and MI, respectively. After adjusting for the effects of other factors, it was found that overweight and poor standing balance in eyes closed condition showed significant associations with RS (Table 3), while overweight, sport participation, moderate degree of initial ankle sprain and poor standing balance in eyes closed condition showed significant associations with PI (Table 4) and moderate degree of initial ankle sprain and poor standing balance in eyes closed condition showed significant associations with MI (Table 5).

Table 3 Logistic regression analysis of factors associated with recurrent sprain.

Variables	Crude OR	95% CI		Adjusted OR	95% CI		*p* value
		Lower	Upper		Lower	Upper	
Overweight	1.088	1.042	1.181	1.083	1.036	1.192	<0.001*
Sport participation	1.280	1.172	1.399	0.845	0.752	1.364	0.234
Living in urban area	1.941	0.957	2.100	1.539	1.361	1.741	0.531
Moderate degree of initial ankle sprain	1.269	1.163	1.384	1.139	0.646	1.617	0.052
Poor standing balance: (closed eye condition)	1.414	1.257	1.591	1.142	1.194	1.311	<0.001*

Notes: *OR: ODDs Ratio, CI: Confidence Intervl, *significance at p<0.05.*

Table 4 Logistic regression analysis of factors associated with perceived Instability.

Variables	Crude OR	95% CI		Adjusted OR	95% CI		*p* value
		Lower	Upper		Lower	Upper	
Overweight	2.120	1.064	3.225	1.229	1.063	2.264	<0.001*
Sport participation	1.905	1.017	3.569	1.192	1.052	3.708	0.013*
Living in urban area	1.444	1.252	1.665	1.093	0.992	1.205	0.056
Moderate degree of initial ankle sprain	1.346	1.206	4.582	1.143	1.038	3.541	0.004*
Poor standing balance: (closed eye condition)	5.725	1.636	20.041	3.476	1.872	6.453	0.006*

Notes: *OR: ODDs Ratio, CI: Confidence Interval, *significance at p<0.05*

Table 5 Logistic regression analysis of factors associated with Mechanical Instability

Variables	Crude OR	95% CI		Adjusted OR	95% CI		*p* value
		Lower	Upper		Lower	Upper	
Overweight	0.199	0.100	0.398	0.968	0.254	3.691	0.962
Sport participation	9.230	4.121	20.672	0.446	0.139	1.423	0.173
Living in urban area	15.190	7.411	31.136	0.861	0.239	3.101	0.819
Moderate degree of initial ankle sprain	3.315	1.372	8.013	1.099	1.027	4.370	<0.001*
Poor standing balance: (closed eye condition)	30.908	14.370	66.480	4.251	1.248	14.485	0.021*

Notes: *OR: ODDs Ratio, CI: Confidence Interval, *significance at p<0.05*

Discussion

The present study found that 69.6% of the typical school-age children had previous ankle sprain(s). Severity of the initial sprain varied from mild to moderate degrees as shown in Table 1. The prevalence was higher than that reported in another community that reported in people aged between 18 and 65 years.[9] However, this prevalence was comparable to that reported in high school athletes (66.8 %) and collegiate athletes (65.2%).[13] In addition, it was found that 27.3% of the children with previous ankle sprain reported re-injury of the same ankle (Table 2).

The factors associated with RS were overweight and poor standing balance in eyes closed condition (Table 3). This is consistent with result of a previous study that obese children experienced a higher incidence of recurrent sprain than those of normal weight.[20] Even though obese children were not included in the present study, high BMI seemed to associate with RS. The positive SLS EC referred to poor standing balance in eyes closed condition in this study which indicated impaired ankle proprioceptors.[21] Therefore, previous ankle sprain may lead to a greater opportunity to have another sprain and develop motor performance problems.

The prevalence of CAI was 36.6% in this study population. This prevalence was higher than that determined in high school athletes (31.1%) and collegiate athletes (18.7%).[13] The different prevalence may be due to the differences in children characteristics that the present research studied in younger children who are not athletes. All children in this study engaged in a sport for recreation only. Therefore, knowledge specific to each sport and advice from sport professionals may be useful for the children to prevent injury and deal with CAI.

To explore the CAI components in more details, it was found that PI was the most common component among the three related components of CAI (Table 2). It was found that the prevalence of PI in the population was 35.3%. A previous research in children with severe ankle injuries reported the prevalence of 31%.[22] The risk of PI was high in children with overweight, sport participation, moderate degree of initial ankle sprain and poor standing balance in eyes closed condition (Table 4), whereas previous research indicated the risk factors for PI included previous ankle injury[22] and obesity[20].

Mechanical instability is the only one related components of CAI that requires physical examinations. The prevalence of MI in the present study was 11.6 % (Table 2). This prevalence was lower than that reported for children with severe ankle trauma (18%)[22] because those with severe ankle sprain were not included in the present study. This finding may be due to the fact that children with severe ankle trauma had impaired tendon and ligaments around the joints which could be detected by the anterior drawer test and talar tilt test. Therefore, they were easily detected as having MI than those without severely impaired structures. The present study also found the higher risk for MI in children with moderate degree of ankle sprain and poor standing balance in eyes closed condition. Therefore, early screenings and preventive managements in typical children are recommended. Additionally, exercises or intervention to

improve proprioceptive sensation for standing balance in children with ankle sprain and poor standing balance should be given to minimize the risk of developing MI.

There is a limitation of this study. This study collected data of typical school-age children using convenient sampling. Since the living area of the children was one of the study factors, normal schools from urban, suburban and rural areas were selected. Although we had children from the three different living areas, generalization of the results may be limited to the sampling communities. Since, children in different types of community may have different lifestyles, further studies to explore multidimensional effects of children's lifestyles on prevalence of CAI are highly recommended.

Conclusion

This study determined the prevalence of CAI and related components in typical school-age children by using interview combined with easy-to use tests. The prevalence of CAI was 36.6%. It was found that 69.6% of the population had previous ankle sprain(s), and 27.3% of them re-sprained of the same ankle. The prevalence of PI and MI was 35.3% and 11.6%, respectively. These outcomes confirmed that CAI and related components could occur in general children population.

The factors associated with RS were overweight and poor standing balance in eyes closed condition. The factors associated with PI were overweight, sport participation, moderate degree of initial ankle sprain and poor standing balance in eyes closed condition. While, the factors associated with MI were moderate degree of initial ankle sprain and poor standing balance in eyes closed condition. These findings raised awareness that early screenings and preventive managements in typical school-age children are necessary. Additionally, early intervention strategies should be developed to prevent prolonged suffering of the related components of CAI.

Conflicts of interests

None

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