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Original Article

Assessment of Anti-oxidation Activities of *Saban* Remedy Extracts

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Abstract

Introduction and Objectives: Oxidative stress arises from an imbalance between reactive oxidant species (ROS) and the body's antioxidant defenses, leading to potential damage to proteins, lipids, and DNA, and increasing mutation risks. Antioxidants are crucial in protecting cells from radicals. Nutrient antioxidants play a significant role in detoxifying ROS, and their deficiency is linked to chronic diseases and cancer. Phytochemical antioxidants, including vitamins C, E, and K, plant pigments like carotenoids, and secondary metabolites such as phenolics and polyphenols, are known for their health benefits. Despite its traditional use as a plant-based medicine for cancer patients at the Arokhayasala Temple, the *Saban* remedy lacks published scientific evidence to support its efficacy. Therefore, this study aimed to evaluate the antioxidant properties of *Saban* remedy using different methods to determine its potential antioxidant benefits.

Methods: To investigate the antioxidant activities of *Saban* remedy and its ingredients, two extraction methods, maceration and decoction, were used. The maceration method involved soaking plant materials in 95% ethanol (3 × 3 days). The extracts were filtered and concentrated with a rotary evaporator under reduced pressure at 45 °C. The decoction method involved boiling the plant materials in water for 15 minutes. Subsequently, part of the solution was filtered using a No.1 Whatman® membrane filter. The residue was boiled by the same process as described an additional two times. All three filtrates were mixed and dried using a freeze dryer. In the same pattern, each plant that constitutes the overall ingredients of *Saban* remedy were individually investigated. The antioxidant activities of these extracts were analyzed using multiple assays, including DPPH radical scavenging assay (DPPH assay), ABTS cation radical scavenging assay (ABTS assay), Ferric Reducing Antioxidant Power (FRAP) assay, and Nitroblue Tetrazolium (NBT) reduction assay. Additionally, the study examined the total phenolic and flavonoid

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contents of these extracts using Folin-Ciocalteu and aluminum chloride methods, respectively.

Results: The aqueous extracts showed a higher percentage yield than ethanolic extracts. However, most ethanolic extracts exhibited higher total phenolic and flavonoid contents than aqueous extracts. Notably, both extracts of *Cassia garrettiana* demonstrated the highest total phenolic and flavonoid contents among the samples. The DPPH radical scavenging assay revealed that the aqueous extracts were the most effective, with lower values of the 50 percentage of effective concentration values (EC_{50}) compared to ethanolic extracts. The *Saban* remedy aqueous extract exhibited strong radical scavenging activity with an EC_{50} of 7.98 ± 0.98 μg/mL, whereas the ethanolic extract had an EC_{50} of 27.92 ± 0.89 μg/mL. Similarly, the ABTS assay showed comparable antioxidant activity between the ethanolic and aqueous extracts, with EC_{50} values of 1.06 ± 0.20 mg/mL and 1.03 ± 0.10 mg/mL, respectively. The FRAP assay results demonstrated that the ethanolic and aqueous extracts of *Saban* remedy had FRAP values of 264.86 ± 10.75 and 301.32 ± 0.46 mg Fe^{2+} equivalent per gram extract, respectively. The aqueous extract exhibited the highest reducing power, indicating its potential as an electron donor to scavenge free radicals and reduce oxidative damage. In contrast, the NBT reduction assay demonstrated that the *Saban* remedy ethanolic extract had higher antioxidant activity than the aqueous extract. This assay measures the ability of antioxidants to reduce superoxide radicals, which are highly reactive oxygen species contributing to cellular damage. The observed higher activity of the ethanolic extract in this assay suggests the presence of ethanol-soluble compounds with strong superoxide scavenging capabilities.

Discussion: Plant polyphenols exhibit significant antioxidant activity by directly reacting with radicals, donating electrons to stabilize them, and preventing cellular damage. Antioxidants derived from plant extracts play a crucial role in neutralizing oxidative stress and reducing the risk of chronic diseases. In this study, both ethanolic and aqueous extracts demonstrated promising antioxidant activities, but their efficacy varied depending on the assay used. Cell-based antioxidant activity involves the defense systems within cells against oxidative stress, and our results indicate that ethanolic extracts are more potent than aqueous extracts in cell-based assays. However, aqueous extracts exhibited superior antioxidant activity in chemical-based assays, as evidenced by lower EC_{50} values in DPPH and ABTS assays. The differences in antioxidant activity across laboratory methodologies arise due to variations in oxidant species and measurement techniques. Moreover, differences between our findings and previous studies may be attributed to differences in plant species, extraction methods, and environmental factors affecting the phytochemical composition of herbs. Geographical location, soil composition, climate, and seasonal variations significantly influence the levels of bioactive compounds in medicinal plants. Therefore, this study indicates that the *Saban* remedy exhibits potent antioxidant activities capable of inhibiting various oxidants or ROS, as demonstrated by the DPPH, ABTS, FRAP, and NBT reduction assays. The aqueous extract displayed stronger radical scavenging activity in DPPH and FRAP assays, while the ethanolic extract showed higher activity in the NBT reduction assay. These findings highlight the importance of extraction methods in determining the antioxidant efficacy of plant-based remedies.

Conclusion and Recommendation: This study indicates that the *Saban* remedy exhibits anti-oxidation activities capable of inhibiting various oxidants or ROS, including the DPPH assay, ABTS assay, FRAP assay, and NBT reduction assay. The findings of this study provide scientific evidence supporting the traditional use of *Saban* remedy for its antioxidant properties.

Key words: Anti-oxidation, Arokayasala, Khampramong Temple, *Saban* remedy, Thai Traditional medicine

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บทคัดย่อ

บทนำและวัตถุประสงค์: ภาวะเครียดออกซิเดชันเกิดขึ้นจากความไม่สมดุลระหว่างสารออกซิแดนท์กับความต้านทานฤทธิ์ต้านออกซิเดชันของร่างกาย ซึ่งนำไปสู่ความเสียหายของโปรตีน ไขมัน และดีเอ็นเอ รวมทั้งเพิ่มความเสี่ยงในการกลายพันธุ์ของดีเอ็นเอ โดยสารต้านอนุมูลอิสระมีความสำคัญอย่างยิ่งในการปกป้องเซลล์จากอนุมูลอิสระ สารต้านอนุมูลอิสระที่ได้จากสารอาหารมีบทบาทสำคัญในการต้านสภาวะเครียดออกซิเดชัน และการขาดแคลนสารต้านอนุมูลอิสระเหล่านี้ ส่งผลให้เกิดโรคเรื้อรังและมะเร็ง สารต้านอนุมูลอิสระจากพฤกษเคมี เช่นวิตามิน C, E และ K หรือสารสีพืช เช่น แคโรทีนอยด์ และสารเมตาบอไลต์ทุติยภูมิ เช่น ฟีนอลิกและโพลีฟีนอล เป็นสารพฤกษเคมีที่มีประโยชน์ต่อร่างกาย ซึ่งตำรับยาสาบาน เป็นยาสมุนไพรที่ใช้เพื่อบรรเทาอาการให้แก่ผู้ป่วยมะเร็งที่อโรคยศาลา วัดคำประมง แต่อย่างไรก็ตามยังไม่มีหลักฐานทางวิทยาศาสตร์ที่ที่เกี่ยวข้องของตำรับยาสาบานถึงฤทธิ์ต้านออกซิเดชัน ดังนั้นตำรับยาสาบานและสมุนไพรในตำรับ จะถูกนำไปศึกษาฤทธิ์ต้านออกซิเดชัน

วิธีการศึกษา: การศึกษาฤทธิ์ต้านออกซิเดชันของตำรับยาสาบานและสมุนไพรในตำรับ ด้วยการสกัดสองวิธี คือการหมักและการต้มน้ำ โดยวิธีการหมัก คือการนำตำรับยาสาบานไปแช่ในเอทานอล 95% เป็นเวลา 3 วัน จำนวน 3 รอบ โดยในแต่ละรอบสารสกัดที่ได้ถูกนำมากรองและทำให้เข้มข้นโดยใช้เครื่องระเหยแบบหมุนภายใต้การลดความดันที่อุณหภูมิ 45 °C ส่วนวิธีการต้ม คือการต้มตำรับยาสาบานในน้ำเดือดเป็นเวลา 15 นาที จากนั้น สารละลายถูกกรองผ่านเมมเบรนของ Whatman® เบอร์ 1 และกากที่เหลือถูกนำไปต้มซ้ำอีกสองครั้ง จากนั้นทั้งสามส่วนที่ถูกกรองจะนำมาผสมกันและทำให้แห้งโดยใช้เครื่องทำแห้งแบบเยือกแข็ง ในทำนองเดียวกันส่วนประกอบของสมุนไพรแต่ละชนิดของตำรับยาสาบานจะถูกนำไปหมักและต้มน้ำเหมือนวิธีข้างต้น จากนั้นสารสกัดทั้งหมดที่ได้จะถูกนำไปทดสอบฤทธิ์ต้านออกซิเดชันด้วยวิธีการต่างๆ ได้แก่ การทดสอบการกำจัดอนุมูลอิสระ DPPH (DPPH assay), การทดสอบการกำจัดอนุมูลแคตไอออน ABTS (ABTS assay), การทดสอบพลังการต้านออกซิเดชันโดยการรีดิวซ์เหล็ก (Ferric Reducing Antioxidant Power หรือ FRAP assay) และการทดสอบการลดลงของ Nitroblue Tetrazolium (NBT reduction assay) พร้อมทั้งตรวจสอบปริมาณฟีนอลิกและฟลาโวนอยด์โดยใช้วิธี Folin-Ciocalteu และวิธีอะลูมิเนียมคลอไรด์ เพื่อประเมินฤทธิ์ต้านออกซิเดชันของตำรับยาสาบานและสมุนไพรในตำรับ

ผลการศึกษา: สารสกัดชั้นเอทานอลของตำรับยาสาบานและสมุนไพรในตำรับ มี %yield ต่ำกว่าสารสกัดชั้นน้ำ และจากการทดสอบปริมาณฟลาโวนอยด์รวมและปริมาณฟีนอลิกรวม พบว่าสารสกัดชั้นเอทานอลของตำรับยาสาบานมีปริมาณฟลาโวนอยด์รวมและปริมาณฟีนอลิกรวมสูงกว่าสารสกัดชั้นน้ำ โดยเฉพาะอย่างยิ่งสารสกัดชั้นเอทานอล

และชั้นน้ำของแสมสารที่แสดงปริมาณฟลาโวนอยด์และปริมาณฟีนอลิกรวมสูงที่สุด และจากการทดสอบฤทธิ์ต้านออกซิเดชันด้วยวิธีการ DPPH assay พบว่าสารสกัดตำรับยาสาบานชั้นน้ำมีประสิทธิภาพดีกว่าสารสกัดชั้นเอทานอล โดยสารสกัดชั้นน้ำมีค่าความเข้มข้นของสารที่มีประสิทธิภาพ 50% (EC_{50}) เท่ากับ 7.98 ± 0.98 μg/mL เมื่อเปรียบเทียบกับสารสกัดชั้นเอทานอล (EC_{50} = 27.92 ± 0.89 μg/mL) และเมื่อทดสอบฤทธิ์ต้านออกซิเดชันด้วยวิธี ABTS assay ของสารสกัดชั้นเอทานอลและชั้นน้ำของตำรับยาสาบาน พบว่า มีค่า EC_{50} ใกล้เคียงกัน คือสารสกัดชั้นเอทานอล มีค่า EC_{50} เท่ากับ 1.06 ± 0.20 และสารสกัดชั้นน้ำ มีค่า EC_{50} เท่ากับ 1.03 ± 0.10 mg/mL และจากผลจากการทดสอบฤทธิ์ต้านอนุมูลอิสระด้วยวิธี FRAP assay พบว่าสารสกัดชั้นเอทานอลและสารสกัดชั้นน้ำของตำรับยาสาบาน มีค่า FRAP เท่ากับ 264.86 ± 10.75 และ 301.32 ± 0.46 mg Fe^{2+} เทียบเท่าต่อกรัมของสารสกัดตามลำดับ แสดงให้เห็นว่าสารสกัดชั้นน้ำมีฤทธิ์ดีกว่าสารสกัดชั้นเทอานอล ซึ่งบ่งชี้ถึงศักยภาพในการเป็นผู้ให้อิเล็กตรอนเพื่อกำจัดอนุมูลอิสระของสารสกัดชั้นน้ำ ในทางตรงกันข้ามจากผลการทดสอบฤทธิ์ต้านออกซิเดชันด้วยวิธี NBT reduction assay พบว่าสารสกัดชั้นเอทานอลมีฤทธิ์ต้านออกซิเดชันดีกว่าสารสกัดชั้นน้ำ ซึ่งการทดสอบ NBT reduction assay เป็นการทดสอบที่วัดความสามารถของสารต้านออกซิเดชันในการลดอนุมูลอิสระซูเปอร์ออกไซด์ ซึ่งเป็นอนุมูลออกซิเจนที่มีความไวสูงและสามารถก่อให้เกิดความเสียหายต่อเซลล์ได้ โดยฤทธิ์ที่ดีกว่าของสารสกัดชั้นเอทานอลในการทดสอบนี้บ่งชี้ถึงการมีอยู่ของสารประกอบที่ละลายในเอทานอลซึ่งมีความสามารถในการกำจัดอนุมูลซูเปอร์ออกไซด์อย่างมีประสิทธิภาพ

อภิปรายผล: สารพฤกษเคมีในพืชคือ Polyphenols มีฤทธิ์ต้านอนุมูลอิสระ โดยการทำปฏิกิริยาโดยตรงกับอนุมูลอิสระ การให้อิเล็กตรอนเพื่อทำให้อนุมูลอิสระมีเสถียรภาพ และป้องกันความเสียหายจากอนุมูลอิสระที่เกิดขึ้นกับเซลล์ สารต้านอนุมูลอิสระที่ได้จากสารสกัดพืชมีบทบาทสำคัญในการลดความเครียดออกซิเดชันและลดความเสี่ยงของโรคเรื้อรัง ในการศึกษานี้ พบว่าสารสกัดทั้งชั้นเอทานอลและชั้นน้ำแสดงฤทธิ์ต้านออกซิเดชันที่มีฤทธิ์ดี อย่างไรก็ตามจะเห็นว่าฤทธิ์ของสารสกัดที่แตกต่างกันขึ้นอยู่กับวิธีการทดสอบที่ใช้ จากผลการศึกษานี้ชี้ให้เห็นว่าสารสกัดชั้นเอทานอลมีฤทธิ์ที่ดีกว่าสารสกัดชั้นน้ำในฤทธิ์ต้านออกซิเดชันในระดับเซลล์ ซึ่งเกี่ยวข้องกับระบบป้องกันภาวะความเครียดออกซิเดชันภายในเซลล์ อย่างไรก็ตามสารสกัดชั้นน้ำแสดงฤทธิ์ต้านออกซิเดชันที่ดีกว่าสารสกัดชั้นเอทานอลในการทดสอบทางเคมี ดังเห็นจากค่าความเข้มข้นของสารที่มีประสิทธิภาพ 50% (EC_{50}) ที่ต่ำกว่าในการทดสอบ DPPH และ ABTS ซึ่งความแตกต่างของฤทธิ์ต้านออกซิเดชันในแต่ละวิธีการทดสอบ เกิดจากความหลากหลายชนิดของอนุมูลออกซิเจนที่ใช้และเทคนิคการวัดผลการทดสอบ นอกจากนี้ความแตกต่างระหว่างผลการศึกษานี้กับงานวิจัยก่อนหน้านี้อาจมาจากความแตกต่างของชนิดพืช วิธีการสกัด และปัจจัยแวดล้อมที่มีผลต่อองค์ประกอบทางเคมีของสมุนไพร เช่น สถานที่เพาะปลูก องค์ประกอบของดิน สภาพภูมิอากาศ และฤดูกาลที่เก็บเกี่ยว ดังนั้นการศึกษานี้แสดงให้เห็นว่า ตำรับยาสาบานมีฤทธิ์ต้านออกซิเดชันที่มีศักยภาพในการยับยั้งอนุมูลอิสระหลายชนิด ดังที่แสดงในการทดสอบ DPPH, ABTS, FRAP และ NBT reduction assay และสารสกัดชั้นน้ำแสดงความสามารถในการกำจัดอนุมูลอิสระได้ดีในวิธีการทดสอบ DPPH และ FRAP ในขณะที่สารสกัดชั้นเอทานอลแสดงฤทธิ์ที่สูงกว่าในการทดสอบ NBT reduction assay ผลการทดสอบเหล่านี้เน้นย้ำถึงความสำคัญของวิธีการสกัดในการกำหนดประสิทธิภาพของฤทธิ์ต้านออกซิเดชันจากสมุนไพร

ข้อสรุปและข้อเสนอแนะ: การศึกษานี้บ่งชี้ว่าตำรับยาสาบาน แสดงฤทธิ์ต้านออกซิเดชันที่สามารถยับยั้งกลุ่มสารออกซิแดนท์ได้หลายประเภท จากการทดสอบด้วยวิธี DPPH, ABTS, FRAP, NBT reduction assays ผลการศึกษานี้เป็นหลักฐานทางวิทยาศาสตร์ที่แสดงถึงฤทธิ์ต้านออกซิเดชันของตำรับยาสาบานและสนับสนุนการใช้ตำรับยาสาบานในทางแพทย์แผนไทย

คำสำคัญ: ฤทธิ์ต้านอนุมูลอิสระ, ตำรับยาสาบาน, การแพทย์แผนไทย, วัดคำประมง, อโรคยาศาล

Introduction and Objectives

Oxidative stress occurs when there is an imbalance between the generation of oxidants or reactive oxygen species (ROS) and the body's ability to neutralize them with antioxidants. This imbalance can result in damage to critical biomolecules, including proteins, lipids, and particularly DNA. Such damage can elevate the risk of mutations. Antioxidants are particularly effective and precise in shielding cells from free radicals[1]. There is substantial evidence supporting the role of nutrient antioxidants in detoxifying reactive oxygen species (ROS). A deficiency in these nutrient antioxidants is linked to the development of various chronic, degenerative diseases, and cancer[2-3]. Phytochemical antioxidants include a range of compounds, such as simple molecule antioxidants like vitamins C, E, and K; plant pigments like carotenoids (β-carotene), xanthophylls, lycopene, and anthocyanins; as well as secondary plant metabolites that range from simple phenolics to more complex polyphenols[3-4]. Antioxidant activity is crucial in cancer treatment and prevention as it neutralizes free radicals and reduces oxidative stress, which contribute to DNA damage, cell mutation, and tumor progression. *Saban* remedy is a plant based herbal medicine administered to cancer patients at Arokhayasala in Khampramong Temple, Thailand[5]. In this study, focusing on antioxidant activity aligns with the traditional use of the *Saban* remedy as a supportive treatment for cancer patients, suggesting it may work indirectly by alleviating oxidative stress and enhancing the body's defense systems. This approach provides measurable evidence to support traditional claims, highlighting the remedy's potential role in cancer management and laying the groundwork for future studies on its anticancer properties.These extracts were investigated for antioxidant activities using various known methodologies. Additionally, the study examined the total phenolic and flavonoid contents of these extracts.

Methodology

1. Plant materials and extraction methods

The plants were collected from Kanchanaburi province, Thailand, in January 2019 and authenticated by the Department of Applied Thai Traditional Medicines, Thammasat University. The vouchered specimens were deposited at the herbarium of Southern Center of Thai Medicinal Plants, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand (Table 1).

The plants were washed, sliced thinly, and dried in a hot air oven at a temperature, not more than 50°C, and ground to a coarse

powder. The plant was extracted by two methods, maceration, and decoction. Then, these ingredients were weighed and mixed into the *Saban* remedy formula. One kg of the dried remedy was macerated with 95% ethanol (3 × 3 days). The extracts were filtered and concentrated with a rotary evaporator under reduced pressure at 45°C. One kg of the dried remedy was boiled in distilled water for 15 minutes. Subsequently, part of the solution was filtered using a No.1 Whatman® membrane filter. The residue was boiled by the same process as described an additional two times. All three filtrates were mixed and dried using a freeze dryer. In the same pattern, each plant that constitutes the overall ingredients of *Saban* remedy were individually investigated.

2. Chemicals and reagents

All the chemicals and reagents used in this study were analytical grade. Folin-Ciocalteu's phenol reagent, quercetin, butylated hydroxytoluene (BHT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), potassium persulfate, 2,2 -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,4,6tri(2pyridyl) 1,3,5triazine (TPTZ), sodium nitrite ($NaNO_2$), aluminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$), nitroblue tetrazolium (NBT), and phorbol-myristate-acetate (PMA) were purchased from Sigma-Aldrich (USA). Iron (III) chloride hexahydrate ($FeCl_3.6\ H_2O$) was purchased from Qrec (New Zealand). Hydrochloric acid (HCl) was purchased from Merck (Germany). Sodium carbonate and sodium hydroxide (NaOH) were purchased from Univar (Australia). Gallic acid was obtained from Thermo Fisher Scientific (Thailand). Aluminum chloride, Dimethyl sulfoxide (DMSO) was obtained from RCI Labscan (Thailand). The human promyelocytic leukemia cell line (HL-60: ATCC® CCL-240™) was purchased from the American Type Culture Collection (ATCC). Iscove's Modified Dulbecco's Medium (IMDM), penicillin-streptomycin (P/S), and phosphate buffer saline (PBS), Fetal bovine serum (FBS), hanks' Balanced Salt Solution (HBSS), and trypsin-EDTA were purchased from Gibco® (OK, USA). Sulforhodamine B (SRB) was purchased from Fluka (Munich, Germany).

3. Chemical analysis of Saban remedy and its ingredients

3.1 Determination of total phenolic content

The total phenolic content was determined according to the Folin-Ciocalteu assay[6]. First, the reaction mixture was prepared using the extract or standard solution, distilled water, and Folin-Ciocalteu's reagent (1:5 with water). Subsequently, 7.5% (w/v) sodium carbonate was added and then incubated at room

temperature for 30 minutes. The absorbance of the blue color was measured at 765 nm. Then, the total phenolic content was calculated according to a gallic acid calibration curve and expressed as milligram gallic acid equivalent per gram extract (mg GAE/g extract).

3.2 Determination of total flavonoid content

The total flavonoid content was analyzed using the aluminum chloride colorimetric method[7]. Briefly, extract or standard solution was mixed with the reaction mixture containing 5% (w/v) $NaNO_2$ and 10% (w/v) $AlCl_3 \cdot 6H_2O$ and further incubated for 5 minutes. Then, 1M NaOH and distilled water were added and incubated for 30 minutes to develop a pink color. The mixture was assayed at 510 nm, and total flavonoid content was expressed as milligram quercetin equivalent per gram (mg QE/g gram).

4. *In vitro* anti-oxidation activities

4.1 DPPH Radical Scavenging Assay (DPPH assay)

The antioxidant activity of these plant extractions was evaluated by the DPPH radical scavenging assay.The assay was described by Yamasaki *et al.*, 1994[8] and utilized with some minor modifications. A 100 μl of sample solution was added into 96 well-micro plates, and then 100 μl of 6×10^{-5} M 2,2-diphenyl-1-picrylhydrazyl (DPPH) in absolute ethanol was pipetted into each well. Afterwards, the plate was incubated in the dark to avoid light for 30 minutes at room temperature. The absorbance was measured at 520 nm using a spectrophotometer. This antioxidant activity testing method used butylated hydroxytoluene (BHT) as a positive control. All tests were determined in triplicate. The 50 percentage of effective concentration values (EC_{50}) were reported as Mean ± SEM of three replicates.

4.2 ABTS cation radical scavenging assay (ABTS assay)

The procedure followed the method of Re *et al.*, 1999[9] with some minor modifications. ABTS cation radical was produced by the reaction between 7 mM of 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) in water and 2.45 mM potassium persulfate (1:1), stored in the dark at room temperature for 16 h before use. ABTS solution was diluted with ethanol to obtain an absorbance of 734 nm. After the addition of 296 μl of diluted ABTS solution to 4 μl of the extracts, the absorbance was measured at 0 minutes after the initial mixing and incubated for 6 minutes. Then, the absorbance was measured at 6 minutes. An appropriate solvent was utilized as the base-

line control in this assay. All the measurements were carried out at least three times. Trolox was used as a standard antioxidant substance. The 50 percentage of effective concentration values (EC_{50}) were reported as Mean ± SEM of three replicates.

4.3 Ferric reducing antioxidant power Assay (FRAP assay)

The procedure followed the method of Benzie and Strain 1996; Pulido *et al.*, 2000; Firuzi *et al.*, 2005[10-12] with some minor modifications. Twenty microlitres of sample solution were added to 96-well microplates. Control solvent was added to the absolute ethanol of the ethanolic extract and distilled water of the aqueous extract. After that, 180 μl of the fresh FRAP reagent was added to 96-well microplates. The sample was kept at room temperature for 8 minutes before being measured for UV absorbance. Its absorbance was measured at 593 nm using a microplate reader. All the measurements were carried out at least three times. Trolox was used as a standard antioxidant reference substance. The values were reported as Mean ± SEM of three replicates. The results were calculated using a Trolox calibration curve and expressed as milligram Trolox acid equivalent per gram extract (mg TEAC/g extract).

4.4 NBT reduction assay

Intracellular superoxide formation was quantified by nitroblue tetrazolium reduction (NBT) assay according to the method of Srisawat *et al.*, 2010 and Mosmann, 1983[13-14]. Briefly, the human promyelocytic leukemia cells (HL-60) were cultivated for seven days in an IMDM medium supplemented with 20% heated fetal bovine serum containing 1.3% DMSO. HL60 was incubated with various dilutions of the extracts and dissolved in HBSS at 37 °C in a 5% CO_2 atmosphere with 95% humidity for 15 minutes. Then, the samples were incubated with ten μg/ml PMA and 1.25 mg/ml NBT solution for another hour. At the end of the incubation time, 2 ml of 1 M HCl was added. After vortexing and centrifugation at 4,000 rpm for 10 min, the precipitate of insoluble formazan deposits was dissolved in 300 μl DMSO. The absorbance was measured at 572 nm. This antioxidant activity testing method used propyl gallate as a positive control. The inhibition against superoxide formation measured by NBT reduction was calculated as a percentage inhibition

5. Statistical analysis

The results of the biological activities are reported as Mean ± Standard Error Mean (SEM) from three replicated experiments.

EC_{50} values were calculated using regression analysis. Mean differences among groups were analyzed by Student's *t*-test. Statistical analysis was conducted by using GraphPad Prism software (CA, USA).

Results

1. Extraction yields and Chemical compounds analysis

All plants were extracted by both the maceration and decoction methods. The percentage yield of all extracts and results of total phenolic and total flavonoids contents are shown in Table 1. The ethanolic extracts showed a percentage yield lower than aqueous extracts. *T. crispa* Forman. had the highest percentage yield of 6.03%, followed by 5.53% of *T. triandra* (Colebr.) Diels. Percentage yield by maceration method showed that *T. baenzigeri* L. had the highest percentage yield of 16.40%, followed by 14.95% of *T. crispa* Forman.

The most ethanolic extracts showed higher total phenolic and flavonoid contents than aqueous extracts. Both extracts of *C. garrettiana* Craib showed the highest amounts of total phenolic. The ethanol extract of *Saban* remedy was determined to have a higher total phenolic than the aqueous extract. Likewise, the highest amounts of flavonoid content were determined in both extracts of *C. garrettiana* Craib. The ethanol extract of *Saban* remedy was shown to have a higher total flavonoid content than the aqueous extract. The results are similar to the total phenolic contents.

2. *In vitro* anti-oxidation activities

Several analytical methods were used to improve antioxidant effectiveness. The results of antioxidant activity were determined by four different test methods: DPPH, ABTS, FRAP, and NBT reduction assay (Table 2). The aqueous extracts were the most effective DPPH radical scavengers. The *Saban* remedy aqueous extract was an effective radical scavenger with an EC_{50} of 7.98 ± 0.98 μg/mL when compared with the ethanolic extract of *Saban* remedy (EC_{50} = 27.92 ± 0.89 μg/mL). The *Saban* remedy ethanolic extract was a less effective radical scavenger than the positive butylated hydroxytoluene (BHT) which showed an EC_{50} = 17.04 ± 0.93 μg/mL. In parallel with these findings, both aqueous extracts of *C. garrettiana* Craib and *P. reticulatus* were also effective radical scavengers compared to BHT as a positive control.

Another antioxidant activity screening method, ABTS radical cation decolorization assay, revealed results like those of the DPPH reaction. The aqueous extracts were observed as powerful ABTS radical scavengers compared to the ethanolic extracts. *Saban* remedy's

Table 1 Scientific name, extraction yield, total phenolic and total flavonoid contents of plant materials.

Herbal plants	Voucher specimen number	Part of used	Extract	%yield	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)
Saban remedy	-	-	95% Ethanol	3.24	147.05 ± 5.44	605.2 ± 63.79
			Aqueous	7.74	101.74 ± 2.46	168.53 ± 5.34
Baliospermum montanum (Willd.) Muell. Arg.	SKP 071 02 13 01	Root	95% Ethanol	2.62	24.82 ± 2.45	41.2 ± 2.89
			Aqueous	7.83	26.12 ± 2.14	82.87 ± 4.62
Carissa carandas L.	SKP 072 03 03 01	Root	95% Ethanol	1.48	26.31 ± 2.66	141.37 ± 25.96
			Aqueous	7.78	35.90 ± 6.84	102.92 ± 12.79
Cassia garrettiana Craib	SKP 034 03 07 01	Wood	95% Ethanol	3.24	656.80 ± 29.81	2451.47 ± 61.35
			Aqueous	4.78	395.71 ± 15.06	853.4 ± 8.5
Croton oblongifolius Roxb.	SKP 071 03 15 01	Stem	95% Ethanol	3.34	51.21 ± 3.75	81.87 ± 6.1
			Aqueous	3.84	61.57 ± 1.37	93.2 ± 7.01
Harrisonia perforate Merr.	SKP 178 08 16 01	Root	95% Ethanol	2.87	97.76 ± 5.07	197.03 ± 4.67
			Aqueous	4.97	151.70 ± 2.27	184.37 ± 7.67
Phyllanthus amarus Schum. & Thonn.	SKP 071 16 01 01	Whole plant	95% Ethanol	3.57	42.29 ± 9.84	94.53 ± 2.33
			Aqueous	9.94	152.42 ± 4.31	134.48 ± 3.13
Phyllanthus reticulatus	SKP 071 16 01 01	Root	95% Ethanol	2.02	130.75 ± 5.20	384.03 ± 11.17
			Aqueous	3.76	193.47 ± 2.92	493.98 ± 14.36
Tinospora crispa Forman.	SKP 114 20 03 01	Stem	95% Ethanol	6.03	29.25 ± 1.96	332.53 ± 2.91
			Aqueous	14.95	25.28 ± 1.60	48.64 ± 10.4
Tinospora baenzigeri L.	SKP 114 20 02 01	Stem	95% Ethanol	3.29	62.09 ± 1.24	259.37 ± 16.74
			Aqueous	16.40	29.39 ± 1.79	56.7 ± 5.48
Tiliacora triandra (Colebr.) Diels.	SKP 114 20 20 01	Root	95% Ethanol	5.53	75.73 ± 1.96	206.53 ± 9.94
			Aqueous	8.09	91.61 ± 7.95	150.31 ± 9.91

ethanolic and aqueous extract demonstrated nearly identical ABTS scavenging with EC_{50} = 1.06 ± 0.20 and 1.03 ± 0.10 mg/mL, respectively. ABTS radical scavenging activity for both *Saban* remedy extracts was comparable with the Trolox (0.21 ± 0.01 mg/mL). Remarkably, the ethanolic and aqueous extracts of *C. garrettiana* Craib also similarly possessed strong activity as demonstrated by an EC_{50} = 0.10 ± 0.02 and 0.30 ± 0.06 mg/mL, respectively.

The FRAP assay measures antioxidants in a sample compared to other assays measuring the inhibition of free radicals. The value expressed from the FRAP assay represents the corresponding concentration of electron-donating antioxidants with the reduction in ferric iron (Fe^{3+}) to ferrous ion (Fe^{2+}). +). The ethanolic and aqueous extracts of the *Saban* remedy exhibited strong reducing power in the Fe III-TPTZ complex, with FRAP values of 264.86 ± 10.75 and 301.32 ± 0.46 mg Fe^{2+} equivalent per gram of extract, respectively. The aqueous extract showed the highest reducing power, indicating its potential as an electron donor to scavenge free radicals. The ethanolic extract of *Saban* remedy showed FRAP values of 106.33 ± 5.01 mg Trolox equivalent per gram extract while the aqueous extract showed FRAP values of 123.32 ± 0.21 mg Trolox equivalent per gram extract. Remarkably, both extracts of *C. garrettiana* Craib had higher antioxidant activity than Saban remedy extracts with FRAP values in both extractions in mg Fe^{2+} equivalent per gram extract and mg Trolox equivalent per gram extract.

The nitroblue tetrazolium test, often referred to as the NBT reduction assay, quantifies ROS produced by leukocytes and spermatozoa. Free oxygen radicals decrease NBT, resulting in the formation of the blue-black chemical formazan. The test is easily accessible and reasonably priced[15]. The extracts were shown to have toxicity in different concentrations by the MTT assay. The extract without significant toxicity was chosen for the NBT reduction assay. The result showed that the *Saban* remedy ethanolic extract had higher antioxidant activity by NBT reduction assay than the aqueous extract. The ingredients contained within *C. garrettiana* Craib (CG) in both extracts revealed the highest antioxidant activity compared to other ingredients. The propyl gallate as positive control showed an EC_{50} valued at 5.13 ± 0.88 μg/mL.

Discussion

Natural phenolic, flavonoid and stilbene chemicals are plant secondary metabolites that have an aromatic ring with at least one hydroxyl group. Because their hydroxyl groups are good electron donors, they can directly contribute to antioxidant activity[16]. Con-

sequently, plant polyphenols exhibit proper antioxidant activity. The chemical base for antioxidant activities is a direct reaction between the sample and radicals, which are stable and generate free radicals. The antioxidants work by donating electrons to these free radicals, stabilizing them, and preventing them from causing cellular damage. Examples of the chemical-based methodologies are DPPH, ABTS, and the FRAP assay. On the other hand, cell-based antioxidant activity refers to the antioxidant defense systems that cells possess to protect themselves from oxidative stress such as the NBT reduction assay. In our case, the results of antioxidant activities through cell-based antioxidant activity demonstrated that ethanolic extracts had more potent activity than aqueous extracts. In contrast, antioxidant activities through a chemical basis showed that the aqueous extracts were the most promising activity against antioxidant activity as values resulted in a lower EC_{50} as assessed by the DPPH and ABTS methods. According to previous published research, the ingredients of *Saban* remedy produced antioxidant activity. Sáez *et al*. reported that the stilbene piceatannol from heartwood's *C. garrettiana* had potent antioxidant capacity in the ABTS assay[17]. Zulkhairi and Ibahim *et al*. revealed that the methanolic and aqueous extracts of *T. crispa* had good antioxidant activity determined by the DPPH and ABTS assay methodology. [18-20]. The *B. montanum's* alcoholic extracts revealed a high percentage of antioxidant activity at 100 μg/ml[21]. *P. amarus's* methanolic extract and phyllanthin also showed antioxidant activities through the DPPH radical scavenging and FRAP assays[22]. The ethanol extract had an IC_{50} value greater than the aqueous extract in *H. perforate* roots[23]. The powder and methanol extract of *P. reticulatus* also had antioxidant activity as measured by the DPPH radical assay[24-25]. Overall, the various methods of evaluating antioxidant activities demonstrate the ability to oxidize, reduce, and stabilize several oxidant species. The various methodological protocols and different oxidants could affect the measurements of radical scavenging activity. The findings in the current studies of antioxidant activities are comparable to previous research, however, the variety of herbs utilized, which are influenced by factors such as geography of cultivation area, season, and time of harvest can affect the herbs' biological activities. Some extracts with low total phenolic content (TPC) and total flavonoid content (TFC) may still exhibit potent antioxidant activities due to the presence of other bioactive compounds or highly active specific phenolics and flavonoids. Antioxidant activity is influenced not only by the quantity of phenolic and flavonoid compounds but also

Table 2 Anti-oxidation activities of selected bitter-tasting plants.

Herbal plants	Solvent	DPPH assay (EC_{50}; ug/mL)	ABTS assay (EC_{50}; mg/mL)	FRAP assay (mg Trolox/g extract)	FRAP assay (mg Fe^{2+}/g extract)	NBT reduction assay (EC_{50}; ug/mL)
Saban remedy	95% Ethanol	27.92 ± 0.89	1.06 ± 0.20	106.33 ± 5.01	264.86 ± 10.75	87.79 ± 4.99
	Aqueous	7.98 ± 0.98	1.03 ± 0.10	123.32 ± 0.21	301.32 ± 0.46	280.97 ± 84.38
Baliospermum montanum (Willd.) Muell. Arg.	95% Ethanol	> 100	7.00 ± 0.32	15.22 ± 0.3	69.36 ± 0.64	> 125
	Aqueous	75.09 ± 9.17	4.49 ± 0.89	30.06 ± 1.03	101.2 ± 2.21	> 1000
Carissa carandas L.	95% Ethanol	> 100	7.01 ± 1.68	25.82 ± 1.22	92.1 ± 2.63	> 1
	Aqueous	65.24 ± 5.62	4.14 ± 0.52	30.52 ± 0.94	102.19 ± 2.02	> 1000
Cassia garrettiana Craib	95% Ethanol	5.28 ± 0.23	0.10 ± 0.02	838.76 ± 9.27	1913.4 ± 15.61	11.89 ± 0.5
	Aqueous	6.28 ± 0.64	0.30 ± 0.06	318.59 ± 10.08	787.99 ± 9.68	> 1
Croton oblongifolius Roxb.	95% Ethanol	> 100	2.08 ± 0.20	20.31 ± 3.16	80.26 ± 6.77	> 500
	Aqueous	29.79 ± 6.38	2.15 ± 0.36	37.78 ± 1.48	117.76 ± 3.18	> 1000
Harrisonia perforata	95% Ethanol	18.09 ± 1.91	8.87 ± 0.83	97.29 ± 4.29	245.45 ± 9.21	101.72 ± 5.86
	Aqueous	10.00 ± 1.55	0.71 ± 0.16	194.52 ± 4.38	454.1 ± 9.4	169.23 ± 10.89
Phyllanthus amarus Schum. & Thonn.	95% Ethanol	18.75 ± 0.57	2.78 ± 0.50	56.57 ± 1.21	158.07 ± 2.59	> 125
	Aqueous	5.51 ± 0.28	0.38 ± 0.12	195.33 ± 1.9	455.83 ± 4.08	274.82 ± 96.39
Phyllanthus reticulatus	95% Ethanol	5.61 ± 0.30	1.10 ± 0.39	175.33 ± 4.06	412.92 ± 8.72	386.8 ± 71.07
	Aqueous	5.79 ± 0.96	0.55 ± 0.06	259.17 ± 4.82	592.83 ± 10.34	240.43 ± 55.15
Tinospora crispa	95% Ethanol	> 100	> 10	32.22 ± 1.83	105.82 ± 3.93	> 125
	Aqueous	70.60 ± 16.53	6.43 ± 1.31	23.89 ± 1.38	87.95 ± 2.97	> 1000
Tinospora baenzigeri	95% Ethanol	56.60 ± 11.69	1.93 ± 0.55	57.08 ± 4.61	159.17 ± 9.88	474.31 ± 20.08
	Aqueous	54.95 ± 24.16	5.14 ± 0.78	27.3 ± 1.12	95.26 ± 2.41	> 1000
Tiliacora triandra	95% Ethanol	26.81 ± 2.53	3.82 ± 0.32	51.12 ± 4.37	146.39 ± 9.38	> 62.5
	Aqueous	13.35 ± 3.28	1.78 ± 0.5	122.56 ± 3.23	299.67 ± 6.93	> 1000
Butylated hydroxytoluene (BHT)		17.04 ± 0.93	ND	ND	ND	ND
6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox)		ND	0.21 ± 0.01	ND	ND	ND
Propyl gallate		ND	ND	ND	ND	5.13 ± 0.88

by their structural characteristics, such as the number and position of hydroxyl groups, which enhance free radical scavenging ability. Additionally, non-phenolic antioxidants, such as alkaloids, terpenoids, and certain vitamins, may contribute to the overall antioxidant activity. Synergistic interactions among various compounds in the extract can also amplify antioxidant effects, even when individual compound concentrations are low. This highlights the complexity of antioxidant mechanisms and suggests that the potency of an extract is not solely dependent on its TPC or TFC but also on the composition and interactions of its diverse bioactive constituents. Regardless, the antioxidant activity data supports the traditional use of the *Saban* remedy for cancer treatment by providing evidence of its potential role in mitigating oxidative stress, a key factor in cancer development and progression. By demonstrating strong antioxidant activity, the extracts of the *Saban* remedy suggest their ability to neutralize free radicals and reduce oxidative damage, which could indirectly contribute to cancer prevention or management. This aligns with its traditional use as a supportive treatment for cancer patients, as reducing oxidative stress can enhance the body's natural defense mechanisms, protect healthy cells during conventional treatments, and improve overall well-being.

Conclusion

In conclusion, the *Saban* remedy antioxidant activities as assessed through DPPH, ABTS, FRAP, and NBT reduction assays was evident. The *Saban* remedy aqueous extract exhibited greater antioxidant activity compared to the ethanolic extract on DPPH and ABTS assays with EC_{50} values of 7.98 ± 0.98 μg/ml and 1.03 ± 0.10 mg/ml, respectively. The aqueous extract of *Saban* remedy demonstrated superior antioxidant activity in the DPPH assay compared to BHT (butylated hydroxytoluene), which was used as the positive control. The FRAP values of SBW also demonstrated a higher value than SBE. Its ingredients exhibited the highest amounts in the FRAP assay in both extracts of *C. garrettiana* Craib. The *Saban* remedy ethanolic extract also showed greater antioxidant activity as measured by the NBT reduction assay than the aqueous extract. Overall, the study finding support the use of *Saban* remedy aqueous extract in folk medicine by evidence based scientific methodologies that it possesses antioxidant properties suitable for health supplements.

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Conflict of interest

The authors declare no conflict of interest

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