Formulation and Phytochemical Profile of a Product Prototype Infused with *Cannabis sativa* Leaves

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Abstract

This study investigated the formulation of a product prototype RMUTI-SKC by using the legal part of cannabis plants (leaves). Five product prototypes were composed of cannabis (leaves), stevia and mint. The herbs were added to improve the taste, smell and physical appearance properties of the product prototype which was yellowish-green. The analysis of the chemical properties found no heavy metals (Cd, As, and Pb) were observed (based on THAI community product standard, and demonstrated a moderate antioxidant activity, total phenolic content and total flavonoid content. The GC-MS profile of crude extracts analysis revealed the phytochemicals based on pharmacological action of essential oil, flavoring substance, anti-inflammatory, antioxidant, anti-bacterial, antidiabetic, antifungal and anti-cancer, respectively. The experimental results showed the pharmacological properties of the product prototype which can be a guideline for applying cannabis plants to other products. Further study on pathogen testing and product aging should be carried out to satisfy the quality of production.

Keywords: Marijuana, Phytochemical, Antioxidant activity, Cannabis, Physiochemical analysis

Introduction

Cannabis (also known as marijuana) has diverse species such as *Cannabis sativa*, *Cannabis indica*, and *Cannabis ruderalis* [1]. Cannabis is a flowering plant that produces resin (Glandular trichome as resin producer, excluding the seeds and leaves when not accompanied by the tops) [2]. In 1961, The United Nations Single Convention on Narcotic Drugs permitted the extraction of cannabis in the form of crude extract and pure compound. The word "*cannabis*" has been used instead of "*marijuana*" due to the narrow definition of the latter in botanical. Cannabis differences in the local name that has been used in the pharmaceuticals and relaxation due to the compounds called cannabinoids. In addition, cannabinoids can also cause negative effects. *Psychoactive cannabinoids can be obtained from*; (a) *Phytocannabinoids* produced from *Cannabis sativa* or *Cannabis indica*; (b) *Endocannabinoids* produced in the brain or peripheral tissue, as a function of neurotransmitters (cannabinoid receptors); and (c) *Synthetic cannabinoids* that are obtained through laboratory synthesis process to have a similar chemical structure to phytocannabinoids or endocannabinoids (Identical biological mechanisms) [3]. The active compounds in cannabis, which come in more than 500 varieties and of which 104 types have been identified, have an impact on the nervous system [4]. The compounds that have been of interest in pharmacological industry are Δ9-tetrahydrocannabinol (Δ9-THC) and cannabidiol (CBD). The potency of cannabis, which affects the nervous system, depends on the THC concentration [5]. Studies investigating the protective properties of CBD have revealed that CBD can counteract the harmful effects of THC [6].

Phytocannabinoids have also been discovered in the genus Radula (liverworts) and Helichrysum (sunflower family) [7]. Although cannabis has been used and studied for a long time, the bio-synthetic process of cannabinoid compounds has recently attracted educational attention. The 1st cannabinoids to be examined were Cannabigerol (CBG, CBGa) [8], which changed to the compounds of THCa, CBDa and CBCa through the synthesis process of oxidocyclase THCa, CBDa or CBCa synthase [9], respectively.

Cannabigerol is a synthetic compound from olivetolic acid (OLA) and geranyl diphosphate (GPP) obtained from polyketide process and non-mevalonate pathways, respectively. The cannabinoid THC, CBD and CBC contains C5 side chain and substituted moiety as C3. The THCVa, CBDVa and CBCVa cannabinoids are produced from the same reactant of divarinolic acid (DVA) and GPP [10].

Terpenoid compounds have excellent absorptivity due to their lipophilic properties thus are transported through the semipermeable membrane to circulatory system. The transported active compounds affect the brain, heart and other organs of the body. *Cannabis sativa* L. chemovars is classified into terpenoids as: D-limonene (the oil from citrus trees influenced sleep and depression in mice and humans), β-Myrcene (anti-inflammatory, analgesic and sedative properties), α-Pinene (anti-inflammatory, antibacterial asthma treatment, and improvement of short-term memory as induced by THC), D-Linalool (Reduce anxiety) and β-Caryophyllene (sesquiterpenoid compound found in *Cannabis sativa* L.), respectively.

The antioxidants in plants are composed of several compounds as ascorbic acid, phenolic compounds and terpenoid compounds. Terpenoids are the main essential oils that act as antioxidants by 3-pathways: 1) Hydrogen transfer, 2) Electron transfer, and 3) Quenching of singlet oxygen. The reactive oxygen species and antioxidant activity of mono-terpene and di-terpene have been studied. Reactive oxygen species (ROS) are produced from the metabolism and cellular response to environmental stress. Factors affecting ROS are insufficient nutrition, irradiation, pathogen, neurodegeneration, cardiovascular deterioration, diabetes and other diseases. Reactive antioxidant molecules prevent free radicals as its primary function, preventing oxidation-related damage. Antioxidant compounds decelerate or combat the oxidation of biological molecules and help to maintain or repair cells via a direct mechanism of free radical scavenging and/or indirect mechanism by increasing the antioxidant concentration. Pharmacological and biological properties that decrease oxidative stress conditions depend on the structures and terpenes derivatives (different liver-related diseases, neurological disorders, coronary artery disease, cancer, diabetes and age). According to the excellent antioxidant properties in cannabis, the herbal tea product has been development by formulation the prototype product infused with *Melissa officinalis* L. and *Stevia rebaudiana* (Bert.).

Mentha is a plant in the *Lamiaceae* family which was described and named by Jussieu 1789. Its flowers are characterized with pronounced lip-like lower petals. Most of Lamiaceae family plants, which are annual or perennial, were first found on Mediterranean Basin and are commonly distributed around tropical areas (the size of the tree is small) [11]. The general Lamiaceae and Verbenaceae are similar and breed as different genus. The 8 sub-genera bred from Lamiaceae family are Ajugoideae, Chloranthaceae, Lamioideae, Nepetoideae, Pogostemonoideae, Scutellarioideae, Teucrioideae and Viticoideae. Nepetoideae is the largest sub-genera of Lamiaceae, accounting for 47 %. The *Lamiaceae* are composed of 260 family and more 7,000 strains. The features identify as a cross-section ofa square trunk, perfect flower composed of 5 petals arranged symmetrically with pollen inside the flower. Normally, the petals of the plants are low and larger than in other location, seeds are dried and hard, an oval appearance of petal and the leaves have strong aroma. The plant is composed of multiple strains such as mints which are used in culinary, pharmaceutical, herbal and decoration [12].

Melissa officinalis L. (Lamiaceae) known as peppermint (lemon balm) is important in the field of medicinal plants and its pleasant fragrance. Peppermint extracts have been commercially used in herbal medicine making, food and cosmetic industry. The main composition of peppermint essential oil is the isomers of monoterpene as neral (α-citral) and geranial (β-citral) which have sedative properties. The smell of *Melissa officinalis* is similar to lemon as it contains Citral essential oil. The smell has been altered by the environment leading to a decrease in the essential oil content. Peppermint oils possess antioxidant, antimicrobial, antibacterial, antifungal, sedative and preservative activities. Trichome of peppermint leaves possess the lowest level of essential oils in the range of 0.02 to 0.40 % which is expensive due to high demand in the market. Plant structure or its metabolism is responsible for minerals and active constituents. The essential oils are composed of a large amount of nutrients from biological synthesis and accumulation in the form of secondary metabolites [13].

Stevia rebaudiana Bertoni (Bert.) is a plant in the family of Asteraceae which is important in for its sweetening compounds known as Steviol glycosides (SGs). The SGs are 300 times sweeter than sucrose and with pharmacological action whereby the extracts enhance body metabolism stability and non-toxicity (a vast biological effect). *Stevia* which is used as sweetener instead of sucrose contains 13 % stevioside, 4 % rebaudioside A, 2 % rebaudioside C and 0.7 % dulcoside A. The SGs, which are widely used for type 2 diabetes patients, people with hypertension and myocardial infarction, are not broken down by metabolic processes. China is the main producer of stevia, producing up to 80 % of the world's output while Japan and Korea have the world's top stevia consumption markets. SGs can be used as food additives and in

beverages. The application of bioactive substances found in stevia leaves, such as vitamins, phytosterols, triterpenes and polyphenols, has been researched for their numerous pharmacological benefits [14].

Stevia rebaudiana (Bert.) is a local herb found between Paraguay and Brazil whose leaves collect sweetness with 0 calories. *Stevia* is useful as anti-inflammatory, anti-hypertensive, it counterattacks tumor expansion, hypoglycaemic, it reduces fat, possesses antioxidant, antimicrobial and anti-fungal activities. *Stevia* is important in the field of trade and science (The Asian strains (China, Japan and Taiwan), North America (United States, Canada) and South America (Paraguay, Brazil, Argentina, Peru and Colombia). The flowering and quality of glycoside compounds are based on radiation, day length, temperature, soil moisture, wind and position of the leaves. The simulated conditions and parameters in cultivation provide nitrogen, nutrient and Glutamine concentration, that affected to the plant growth [15].

Steviol glycosides are the natural compounds extracted from *Stevia rebaudiana* (the molecule's sweet taste)*.* Currently, more than 20 SGs compounds have been isolated and structurally identified. Stevia has been used to control the weight of overweight populations and blood sugar. In addition, SGs are used to prevent abnormal fat burning procedure and some diseases such as diabetes mellitus and cancer. However, the effectiveness of SGs is based on the extraction and purification process. The amount of SGs extracted depends on a suitable organic solvent (such as ethanol, methanol, acetone, chloroform and petroleum ether), temperature (60 to 100 °C), and drying time (such as 30 min to 24 h), with extracted SGs in the range of 2 - 35 % [16].

The leaves of *Camellia sinensis*, commonly known as tea, has been the 2nd most popular beverage for over 50 years. The plant originated from Southeast Asia and is currently cultivated in more than 30 countries with a production of 3 billion kg each year. Generally, tea is produced based on a simple process and popularly consumed as 78 % of black tea in Western Countries, 20 % of green tea in Asian Countries and 2 % of oolong tea in China (partial fermentation) [17,18]. This study explored chemical constituents and pharmacological effects of cannabis plants and herbs to formulate product prototype of herbal tea. The physiochemical analysis of the formulation prototype of herbal tea contained cannabis leaves, mint leaves and stevia. This study might act as a guideline for the production and quality control of herbal products containing cannabis plant.

Materials and methods

Plant material
Cannabis leaves were provided by the cannabis greenhouse, Rajamangala University of Technology Isan, Sakon Nakhon Campus, which is legally licensed to produce (grow). Mint leaves and stevia were purchased from a local herbal pharmacy.

Preparation of herbal samples
The prototype of herbal tea composing of cannabis leaves was developed from the research of Díaz-Montes *et al*. [16]. The formulation prototypes were composed of the cannabis leaves, mints leaves and stevia. The plant materials were cleaned with fresh water and dried by Solar Drying Plant at 45 °C for 8 - 9 h, then roasted with stove at 90 - 120 °C for about 3 min. The dried plants were ground to powder with a 2 rollers herbal grinder and sifted with 80 mesh gliding sieves, then dried with hot air incubator at 45 °C for 48 h and 36 °C 24 h, respectively.

Formulation of herbal tea prototype
Five prototypes of herbal tea were formulated with 3 complementary herbs as cannabis leaves, mint leaves and stevia, which composed 1, 3, 5, 7 and 9 % w/w of cannabis leaves modified from the research of Srisopa and Wongkrajang [19] (**Table 1**).

Table 1 Formulation details of herbal tea prototype.

Composition	Formulation				
	1	**2**	**3**	**4**	**5**
Cannabis (g)	1	3	5	7	9
Mint (g)	92	90	88	86	84
Stevia (g)	7	7	7	7	7

Color analysis

The powder of herbal tea prototype color was analyzed based on the system of CIE L*a*b* using the spectrometer Hunter Lab (ColorFlex, Hunter, angle at 10 °, light source as D65). Five formulations, 3 of which were independently selected and analyzed (n = 3) [20].

Extraction process

The formulation prototype extraction was performed following the method derived from the research of Kornpointner *et al*. [21], that used the maceration process. Each prototype formulation was extracted by 95 % ethanol (1:10 %w/v) for 7 days (n = 3). The solvent was removed by rotary evaporator at 60 °C 70 rpm. The crude extracts were dried again with hot air oven 45 °C for 24 h. The extracts were subsequently analyzed by GC-MS, AAS and antioxidant, Total Phenolic content and Total Flavonoids content were determined.

Chemical analysis
Gas chromatography-mass spectrometry (GC-MS)

The GC-MS was used to analyze the crude extracts of formulation prototype at concentration of 0.2 mg/mL, using Shimadzu GCMS-QP2020 Ultra mass selective detector coupled with Shimadzu QP2020 Ultra gas chromatograph, equipped SH-Rtx-5MS capillary column (30 m×0.32 μm, film thickness 0.25 μm). The measured conditions were modified from the method of Jemmali *et al*. [22] as follows: Carrier gas, helium with flow rate of 1.37 mL/min; column temperature starting at 50 °C, 50 - 140 °C (5 °C /min), constant temperature of 140 °C for 15 min; 140 - 200 °C (3 °C/min) then isotherm for 10 min; 200 - 260 °C (15 °C/min). Injector and detector temperatures was 250 °C and a split ratio of 1:50. The electron impact voltage was set at 70 ev. The identification of chemical constituents was carried by comparing their retention index with Library NIST17.

Atomic absorption spectroscopy (AAS)

The analysis of herbal powder, was conducted by an Atomic Absorption Spectrophotometer (AAS: Shimadzu AA-7000). The total Cd (228.8 nm) and Pb (217.0 nm) contents were carried out on Flame Atomic Absorption Spectroscopy (FAAS) and the total contents of As (193.7 nm) was done using Graphite Furnace Atomic Absorption Spectroscopy (GFAAS) [23]. One g of each ground herb was weighted and transferred to a container with 100 and 12 mL of 65 % HNO_3 were added slowly, and then a subsequent 4 mL of 37 % HCl were gradually added. The containers were placed on a heater until the mixture appeared clear. The samples were then cooled and transferred to 100 mL volumetric flask and then the level of the sample solution was adjusted to a volume of 100 mL [24]. The standard solution of Cd, Pb and As were prepared with acidic solvent (12 % v/v HNO_3, 4 % v/v HCl and 84 % v/v DI-water) (**Table 2**).

Table 2 Concentration of standard solution: Cd, Pb and As.

Standard Metal	Concentration						
As (ppb)	10	15	20	25			
Cd (ppm)	0.05	0.10	0.20	0.30	0.40	0.50	0.60
Pb (ppm)	0.50	2.50	5.00	10.00	15.00	20.00	

Method validation was developed from Adamu *et al*. [25]. This was evaluated by measuring several analytical figures, namely linearity and range, precision [26], sensitivity which is expressed by determination of limit of detection (LOD) and limit of quantitation (LOQ), and accuracy.

Determination of antioxidant activity

To determine the antioxidant activity of the herbal tea prototypes, DPPH (2,2-diphenyl-1-picrylhydrazyl) was used to evaluate the free radical scavenging ability following a modified method of Singh *et al*. [27]. The sample concentrations were prepared as 5, 10, 15, 20, 25, 50, 75 and 100 μg/mL with 95 % Ethanol as solvent. Three milliliters of 0.1 mM DPPH were added to a container with 0.1 mL of each sample concentration. The mixtures were then kept at room temperature in the dark for 30 min. The absorbance was measured at 517 nm compared to a blank (DPPH solution without sample) (n = 3). Ascorbic acid was used as the standard antioxidant. The antioxidant activity was calculated with the equation described by Oubihi *et al*. [28].

Determination of total phenolic content

The total polyphenolic content was measured according to the literature [29]; 0.5 mL the of sample extract was added in to a container with 2.5 mL Folin-Ciocalteu reagent (1/10; DI as solvent) then 4 mL of 7.5 % (w/v) sodium carbonate were added. The mixture was then incubated for 30 min in the dark at 45 °C. The absorption was measured at 760 nm. Gallic acid was used as a standard to prepare a calibration curve (2.5 to 250 μg/mL). The results were expressed as mg of gallic acid equivalent (GAE) per gram of extract (mg GAE/g E).

Determination of total flavonoids content

Total Flavonoids content of samples were measured using the method described by Chen *et al.* [30]. Briefly, 2.4 mL of the sample solution (2 mg/mL), 0.3 mL of $NaNO_2$ (0.05 g/mL) were mixed and reacted with 4 mL of NaOH (0.04 g/mL) for 6 min at room temperature. The mixture was then incubated for 15 min for further reaction. Finally, the absorbance was measured at 510 nm against a reagent blank. A calibration curve was prepared using quercetin and the flavonoid content of each sample was shown as quercetin equivalent (mg QE/g dry weight).

Statistical analysis

The data were expressed as mean values ± standard deviation for each measurement and analyzed by means of analysis of variance (1-way ANOVA). A probability of $p < 0.05$ indicates that the values are considered statistically significant.

Results and discussion

Color of herbal tea prototype

Color is one of the properties that indicate product quality. The color of herbal tea prototype was analyzed with a color meter (Hunter Lab; 10 °, light source: D65) [29]. The color analysis of the samples was presented in the system of CIE L*a*b*, revealing that the herbal tea composition significantly influenced all color parameters (L*, a* and b*), as shown in **Table 3**.

Table 3 Color analysis of herbal tea prototype in the CIE L*a*b* system.

| Sample	L* ($\overline{X} \pm$ S.D.)	a* ($\overline{X} \pm$ S.D.)	b* ($\overline{X} \pm$ S.D.)
Cannabis	48.943 ± 0.015	-1.680 ± 0.010	26.473 ± 0.006
Stevia	56.573 ± 0.006	-1.717 ± 0.006	26.517 ± 0.006
Mint	45.550 ± 0.000	0.657 ± 0.006	19.440 ± 0.010
Can1 %	47.627 ± 0.006	0.230 ± 0.000	19.493 ± 0.023
Can3 %	48.740 ± 0.000	0.077 ± 0.006	20.310 ± 0.010
Can5 %	48.180 ± 0.000	0.100 ± 0.010	20.377 ± 0.006
Can7 %	48.270 ± 0.000	0.247 ± 0.012	20.893 ± 0.006
Can9 %	47.843 ± 0.006	0.202 ± 0.006	20.723 ± 0.012
F	758556.743	37130.286	209831.238
p-value	$0.000^\dagger$	$0.000^\dagger$	$0.000^\dagger$

†Between groups within a column: 1-way ANOVA, Post Hoc Tests; $p < 0.05$.

Average color and lightness (L* a* and b*) values in the raw materials of herbs (powder of cannabis, stevia and mint) ranged between 45.550±0.000 - 56.573±0.006, -1.717± 0.006 - 0.657±0.010 and 19.440±0.010 - 26.517±0.006, respectively. The color values (L* a* and b*) of herbal tea prototype ranged between 47.627 - 48.740, 0.077 - 0.247 and 19.493 - 20.893, indicating significant differences ($p < 0.05$).

Identification of compounds by GC-MS

GC-MS was used to analyze the present active constituents of the extracts from the herbal tea prototype. The metabolites were characterized according to retention times (**Figure 4**) and the corresponding mass spectrometric data is presented in **Table 4**.

Chromatograms (**Figure 4**) demonstrated the chemical analysis results of prototype products containing cannabis infused (leaves) by using GC-MS (GC-MS, 70eV: TIC). The chemical constituents were analyzed with Library NIST17 and essential oil, flavoring substance, anti-inflammatory, antioxidant, anti-bacterial, antidiabetic, antifungal and anti-cancer elements were found, corresponding to literature. The compounds in the formulation prototype infused with 1 - 9 % cannabis (**Table 4**) composed of 5.58 % Propane, 1-(1-ethoxyethoxy)-, 3.85 % Propane, 1,1-diethoxy-, 1.60 % Butane, 1,1-diethoxy-, as flavoring substances [37], and 1.72 % 1-Butanol, 3-methyl- acetate as essential oil [32]. The compound Neophytadiene at 30.33 % possessed anti-inflammatory and antibacterial properties [33], and 1-Pentadecyne at 8.08 % had antidiabetic [34], antifungal [35], and anti-cancer properties [36]. The formulation prototypes infused with 3, 5, 7 and 9 % w/w cannabis were found to have chemical consistency consisting of propane, 1-(1-ethoxyethoxy)-, propane, 1,1-diethoxy-, butane, 1,1-diethoxy- and neophytadiene with differentcomposition percentage. Butanol, 3-methyl-, acetate was analyzed from the crude extracts of 1 % w/w similarly to the formulations of 5 and 9 % w/w cannabis.
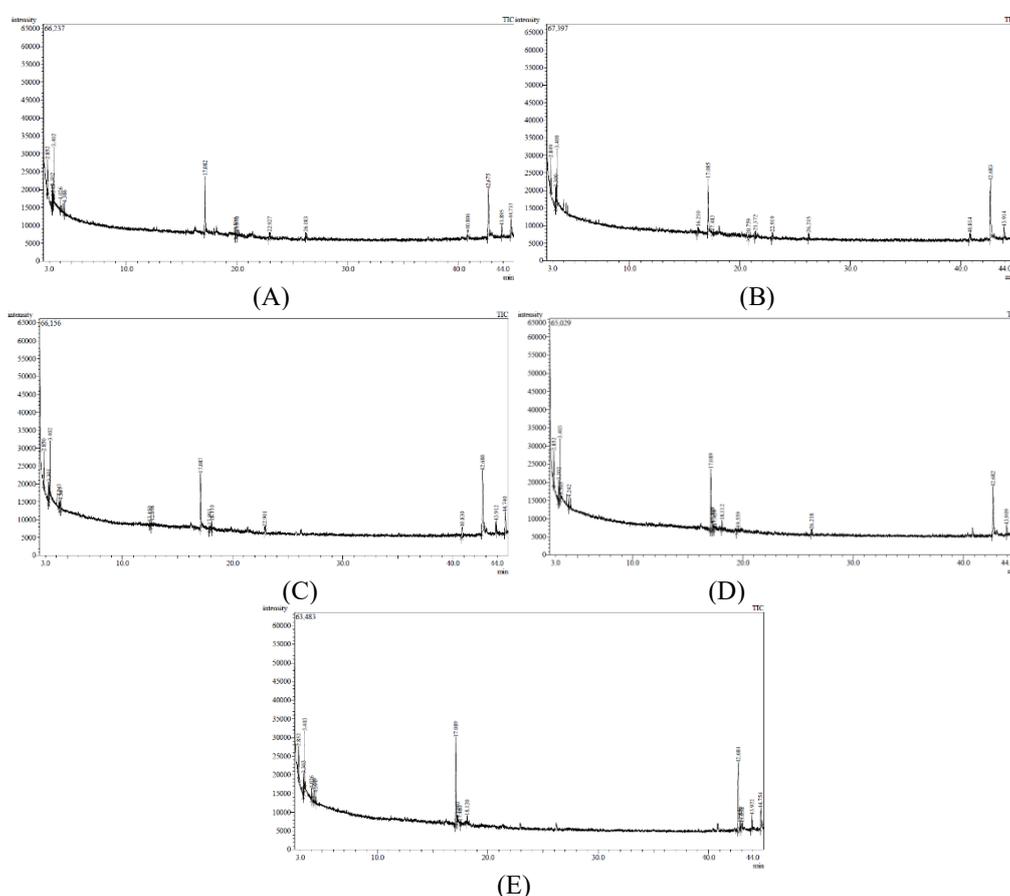
Figure 4 GC-MS chromatogram of herbal tea prototype; (A) 1 % cannabis; (B) 3 % cannabis; (C) 5 % cannabis; (D) 7 % cannabis, and (E) 9 % cannabis.

The list of chemical constituents identified in the formulation prototype infused with 3 % w/w cannabis are the compound 3-Butenoic acid, ethyl ester (1.90 %) which was reported in 2014 as volatile organic compounds [36], with medicine component [38], was analyzed. Citronellyl butyrate (10.67 %) was confirmed in the formulation prototype infused with 5 % w/w cannabis reported as essential oils [39], and plant-derived insecticide [40]. The 3-Oxatricyclo [4.1.1.0 (2,4)] octane, 2,7,7-trimethyl- at 24.59 % from the crudes extracts of 9 % w/w cannabis formulation is used as flavoring substance in food and the pharmaceutical industry [41]. The preliminary GC-MS results confirmed that the chemical constituents and

phytochemical properties of the product prototype were beneficial application for foods and health care products.

Table 4 List of chemical constituents identified in the formulation prototype infused with cannabis by GC-MS analysis.

Compound	Formula	Peak Area %					Pharmacological Action	Ref.
		1 % can	3 % can	5 % can	7 % can	9 % can		
Propane, 1-(1-ethoxyethoxy)-	$C_7H_{16}O_2$	5.58	4.29	4.36	5.46	3.86	Flavouring Substances	[37]
Propane, 1,1-diethoxy-	$C_7H_{16}O_2$	3.85	3.04	3.11	3.74	2.87		
Propane, 1-(1-ethoxyethoxy)-	$C_7H_{16}O_2$	9.92	11.22	9.20	13.14	8.15		
Butane, 1,1-diethoxy-	$C_8H_{18}O_2$	1.60	nd	nd	1.84	1.30		
1-Butanol, 3-methyl-, acetate	$C_7H_{14}O_2$	1.72	nd	2.01	nd	1.31	Volatile compound Aroma substances	[32]
3-Butenoic acid, ethyl ester	$C_8H_{18}O_2$	1.90	1.90	nd	nd	nd	Volatile compound, Aromatic component medical constituents	[36,38]
Neophytadiene	$C_{20}H_{38}$	30.33	33.04	32.88	27.08	34.88	Anti-inflammatory efficacy, Antibacteria Activities	[33]
1-Pentadecyne	$C_{15}H_{28}$	8.08	nd	nd	nd	nd	Essential oil, Antioxidant, Antibacterial, Antifungal, Antidiabetic, Anti-inflammatory, Anti-cholinesterae Volatile Organic Compounds, Hypocholesterolemi Anticancer, Anticoronary	[35,36]
Citronellyl butyrate	$C_{14}H_{26}O_2$	10.67	nd	10.67	nd	nd	Essential oil Insecticidal Activity	[39,40]
3-Oxatricyclo [4.1.1.0(2,4)] octane, 2,7,7-trimethyl-	$C_{10}H_{16}O$	24.59	nd	nd	nd	24.59	Essential oil, Perfumery, Food, Flavouring and pharmaceutical industry	[41]

Note: nd is not detected.

Heavy metal analysis by AAS

Heavy metal concentration was determined by comparing the samples with the standard substances of heavy metals. The Standard Calibration Curve of heavy metals, cadmium (Cd), lead (Pb) and arsenic (As) which was more than 0.9 of the curves gave regressions (coefficients of determination: R^2) an indication of the reliability of the curves [25]. The equations of calibration curves of the standard heavy metals were are seen in **Table 5**. The concentration of heavy metals of the raw materials (the composition of formulations prototype) is demonstrated in the **Table 6**.

Table 5 Calibration curves of standard heavy metals.

Metal	Cd (ppm)	Pb (ppm)	As (ppb)
Equation	y = 0.6872× + 0.0017	y = 0.0215× + 0.0042	y = 0.0086× + 0.0051
R^2	0.999	0.999	0.999
SD	0.151	0.166	0.146
LOD	0.010	0.005	−0.335
LOQ	0.026	0.136	1.298

Table 6 Concentration of heavy metals in the composition of herb materials.

Sample	Cannabis			Mint			Stevia		
	Conc.	**SD**	**%RSD**	**Conc.**	**SD**	**%RSD**	**Conc.**	**SD**	**%RSD**
Cd (ppm)	0.014	$5.80E^{-05}$	0.825	−0.009	$3.00E^{-04}$	4.286	0.009	$4.00E^{-04}$	5.774
Pb (ppm)	0.057	$5.80E^{-05}$	0.215	0.014	$1.00E^{-04}$	0.373	0.038	$1.50E^{-04}$	0.570
As (ppb)	12.989	5.68775	72.599	3.586	1.77186	22.506	1.172	1.9336	24.565

The concentration of Cd (Cannabis = 0.014 ppm, Mint = –0.009 ppm and Stevia = 0.009 ppm) and Pb (Cannabis = 0.057 ppm, Mint = 0.014 ppm and Stevia = 0.038 ppm) the formulation prototype (**Table 6**) were below the LOQ (Cd = 0.026 ppm and Pb = 0.136 ppm) (**Table 5**), which indicated the absence of Cd and Pb [43]. The relative standard deviation percentage (%RSD) as ≤ 15 % revealed the accuracy of the results which were consistent with Kamunda *et al*. [31]. An analysis of the composition of the formulations prototype (Cannabis = 12.989 ppb, Mint = 3.586 ppb and Stevia = 1.172 ppb) presented a relative standard deviation percentage (%RSD) of ≤ 16 % in the range of ppb representing acceptable experimental results, which corresponded to Eka *et al*. [44]. Gonzalez and Herrador configured the %RSD of the same concentration range of 11 % which is an acceptable value [45]. The %RSD of As analysis range were moderate but below the Thai Industrial Standards Institute. According to the Thai Industrial Standards Institute, assigned values are as lead (Pb ≤ 0.5 ppm), cadmium (Cd ≤ 0.3 ppm) and arsenic (As ≤ 0.2 ppm) [42]. The results of heavy metals concentration of the prototype which h were below the limit of the Thai Industrial Standards Institute indicated the absence of heavy metal residues. The less of heavy metal residues in herb materials demonstrated that suitable for using in making products.

Antioxidant activity
Free radicals which generate oxidative stress are produced by oxidation reaction. The substances that can inhibit or slow down oxidation reactions are referred to as "Antioxidants". The antioxidant analysis was carried out with the process of DPPH assay which eliminates DPPH radical in the herbal extracts (DPPH scavenging activity) [27]. Antioxidant tests were performed by the comparison of ascorbic acid (as standard). The antioxidant efficiencies result of the prototype formulations are shown in **Table 7**. It was found that all prototypes demonstrated statistically higher antioxidant activity than standard ascorbic substances (IC_{50} = 0.119 mg/mL), with significant differences at $p < 0.05$. Phongpaichit *et al*. [46], reported the antioxidant profile of Maja Fruit (*Aegle marmelos* (L) Carrea) with high performance activity (IC_{50} ≤ 50 mg/mL), moderate performance activity (IC_{50} as value of 100 - 150 mg/mL) low (IC_{50} as 151 - 200 pg/mL). The antioxidant activity classified as lowest was IC_{50} ≥ 200 mg/mL. Therefore, the experiment results of the prototype products showed a high level of antioxidant capacity (IC_{50} ≤ 50 mg/mL), higher than standard ascorbic substances. The high performances antioxidant activity of the prototype products that can evaluated by a dietary supplement.

Table 7 Antioxidant activity test results of the prototype formulations.

Sample	Equation	R^2	SD	IC_{50} (µg/mL)
Ascorbic	$y = 0.4734 \times - 6.5603$	0.9927	0.4514	94.3095[a]
1 % Can	$y = 0.7291 \times + 6.9879$	0.9892	0.9928	58.9934[a]
3 % Can	$y = 0.6123 \times + 10.424$	0.9906	1.3419	64.9354[a]
5 % Can	$y = 0.7077 \times + 5.0009$	0.9934	1.0966	63.5735[a]
7 % Can	$y = 0.5041 \times + 7.9074$	0.9905	1.6890	83.5005
9 % Can	$y = 0.5637 \times + 3.3782$	0.9892	1.6426	82.7067
F				401.134
***p*-value**				0.000[†]

[†]Between groups within a column: 1-way ANOVA, Post Hoc Tests; $p < 0.05$.

Total phenolic content

Analysis of the total phenolic content of the prototype formulation extracts (1, 3, 5, 7 and 9 % w/w cannabis) was performed by using Folin Ciocalteu's method with gallic acid as a standard substance ($R^2 = 0.9986$). The results revealed the total phenolic values were in the range of 0.2622, 0.3163, 0.2855, 0.3618 and 0.1302 (mg GAE/g dry material) of 1, 3, 5, 7 and 9 % by mass of cannabis (**Table 8**), with a statistically significant difference at $p < 0.05$. Phenolic compounds have redox properties which possess an excellent antioxidant ability [47]. The free radical scavenging ability of hydroxyl moieties and the concentration of phenolic compounds affect the antioxidant activity [48]. Ahmed *et al.* [49], described the results of the antioxidant potential of *Cannabis sativa* L. leaves, which were extracted with methanol as solvent, to have 36.42 mg gallic acid equivalent GAE/g, which is a good antioxidant ability. Phenolic compounds which are naturally derived, indicate the ability to inhibit diabetes and reduce oxidative stress [50].

Table 8 phenolics and flavonoids content of ethanolic extract of the prototype formulations.

Cannabis	Phenolic Content (mg of gallic acid equivalent/g DW)	Flavonoid Content (mg of Quercetin equivalent/g DW)
1 %	0.2622 ± 0.0003	0.3304 ± 0.0049[b]
3 %	0.3163 ± 0.0005	0.3218 ± 0.0039[b]
5 %	0.2855 ± 0.0003	0.3229 ± 0.0166[b]
7 %	0.3618 ± 0.0009	0.3331 ± 0.0000[b]
9 %	0.1302 ± 0.0004	0.3217 ± 0.0029[b]
F	33637.255	8.447
***p*-value**	0.000[†]	0.003[†]

[†]Between groups within a column: 1-way ANOVA, Post Hoc Tests; $p < 0.05$.

Total flavonoids content

The total flavonoid compounds of prototype products containing cannabis leaves (1, 3, 5, 7 and 9 % w/w cannabis) were measured. The values were 0.3304±0.0049, 0.3218±0.0039, 0.3229±0.0166, 0.3331±0.0000 and 0.3217±0.0029 mg of Quercetin equivalent/g dry weight, respectively (**Table 8**). The total flavonoid compounds showed a tendency to decline according to cannabis percentage of 7, 1, 5, 3 and 9, respectively, with statistically significant differences at $p < 0.05$. Drinic *et al.* [51], described the extraction of adult plants with 50 % ethanol provides the best performance for important substances in a

group of cannabinoid compounds (5.21 as mg of catechin equivalent per g dry weight hemp (mg CE/g dw)). Terpenes and phenols condense to become cannabinoids in the glands that cover the plant's above-ground portion [52]. The extraction in this experiment was done with ethanol solvent which highly influenced the total flavonoid content. The total amount of flavonoid compounds of peppermint was found to be high at 45.06 mg CE/100 g DW [53]. Flavonoids in plants have a high antiviral ability and prevent diseases caused by free radicals [54]. The results of the experiments showed that the total amount of flavonoid compounds of the prototype product was high due to the medicinal plants, namely cannabis leaves, stevia and mint. Each herb contains a high total amount of flavonoid compounds, which affects the total flavonoid content of the product and represents the ability to have a beneficial effect on health.

Conclusions

The formulation of the product prototype was composed of cannabis leaves, stevia and peppermint leaves, which resulted in a yellowish-green appearance material. GC-MS analysis of the prototype product demonstrated the substances to have antioxidant effect, anti-inflammatory, anticancer, and antimicrobials properties, and essential oils. The results of GC-MS profile confirmed that the phytochemical properties of the product prototype were beneficial. The residues of heavy metals (Cd, As and Pb) were not found in the samples which met lower than the level of Thai Industrial Standards Institute (Pb $\leq$ 0.5 ppm, Cd $\leq$ 0.3 ppm and As $\leq$ 0.2 ppm). The prototype products showed a high level of antioxidant capacity ($IC_{50} \leq 50$ mg/mL), a high content of total phenolic compounds (7 % cannabis: 0.3618 ± 0.0009 mg of gallic acid equivalent/g DW) and total flavonoid compounds (7 % cannabis: 0.3331 ± 0.0000 mg of Quercetin equivalent/g DW), respectively. For greater integrity of the research results, further tests for pathogens, shelf life, satisfaction, as well as health effects on the consumers should be carried out.

Acknowledgements

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