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ABOUT THE JOURNAL

Journal of Functional Food and Nutraceutical (JFFN) is an official journal of Perhimpunan Penggiat Pangan Fungsional dan Nutrasetikal Indonesia or Indonesian Society for Functional Food and Nutraceutical (P3FNI-ISFFN) that has been established in collaboration with Research Center for Food and Health Swiss German University (RC F&H SGU). JFFN publishes review and research result on frontier research, development, and application in the scope of functional food and nutraceuticals. The journal is expected to bring together all stakeholders in relation to the food ingredients and nutraceuticals.

Scope of the journal Include:

- Interdisciplinary approach of food technology, food nutrition, and health
- Plant bioactive; dietary fiber, probiotics; functional lipids; bioactive peptides; vitamins, minerals and botanicals and other dietary supplements.
- Nutritional and technological aspects related to the development of functional foods and nutraceuticals.
- Food digestion, bioavailability, mechanism, efficacy, and safety of food ingredients and nutraceuticals.
- Food product development with health benefit
- Characterization of healthy foods and functional constituents
- Preparation of natural and synthetic ingredients for use in foods and supplement
- Effects of processing (including packaging and storage) on functionality and improvement of product quality; verification, quality control and traceability of natural and synthetic functional food ingredients and nutraceuticals.
- The regulatory aspects of functional foods and related issues e.g. labelling, substantiation of health claims are also of interest together with those dealing with the value creation on the food chains based on the nutritional/healthy aspects.

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TINJAUAN MANFAAT BUNGA TELANG (CLITORIA TERNATEA L.)
BAGI Kesehatan Manusia

Abdullah Muzi Marpaung
Department of Food Technology, Swiss German University, Indonesia, 15143

ABSTRACT

All part of the butterfly pea (Clitoria ternatea) plant reported having a various positive effect on human health. The blue petal, in particular, shows a wide range of functional activity including as an antioxidant, antidiabetic, antiobesity, anticancer, anti-inflammatory, and antibiotic. The hydrophilic phase of butterfly pea flower extract contains flavonol glycosides, anthocyanins, flavones, flavonols, phenolic acids, and cyclotides. Meanwhile, the terpenoids, alkaloids, and fatty acids were found in the lipophilic phase of butterfly pea flower extract. The proven health benefits and the wide range of the type of bioactive compounds promote butterfly pea flower as the source of functional food and nutraceuticals. However, a series of intensive research, including the clinical trial, is still needed.

Keywords: Butterfly pea; Clitoria ternatea; functional food; nutraceutical.

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Kata kunci: Bunga telang; Clitoria ternatea; nutrasetikal; pangan fungsional.
PENDAHULUAN


Bunga Telang Dalam Pengobatan Tradisional


TINJAUAN MANFAAT BUNGA TELANG (CLITORIA TERNATEA L.)
BAGI KESEHATAN MANUSIA

Marpaung, A.M.

Gambar 1. (Kiri) Tanaman telang, (kanan atas) bunga telang, (kanan bawah) bagian-bagian bunga telang

tradisi pengobatan India. Di antaranya disebutkan manfaat telang (i) untuk mengobati insomnia, epilepsi, disentri, keputihan, gonorrhea, rematik, bronkitis, asma, maag, tuberkulosis paru, demam, sakit telinga, penyakit kulit seperti eksim, impetigo, dan prurigo, sendi bengkak, kolik, sembelit, infeksi kandung kemih, asites (akumulasi kelebihan cairan pada rongga perut) (ii) untuk memperlancar menstruasi, melawan bisa ular dan sengatan kalajengking, (iii) sebagai antiperiodik (obat untuk mencegah terulangnya penyakit kambuhan seperti malaria), obat cacing, pencahar, diuretik, pendingin, pemucu mual dan muntah sehingga membantu mengeluarkan dahak bronkitis kronis, dan stimulan seksual. Sebagai tambahan, oleh masyarakat Arab Saudi daun, biji dan bunga telang dimanfaatkan untuk mengobati penyakit liver atau hati (Al-Asmari et al., 2014). Di Madagaskar daun telang digunakan untuk meredakan nyeri sendi (Jain et al., 2003). Di Myanmar campuran jus bunga telang dan susu digunakan untuk menyembuhkan sakit mata (DeFilipps & Krupnick, 2018). Sementara itu di Indonesia, khususnya masyarakat Betawi, bunga telang digunakan untuk membuat jernih mata bayi.

Dalam sistem pengobatan kuno India (Ayurveda), telang tergolong herbal yang penting. Hal ini dapat terlihat pada nama yang diberikan kepada telang dalam Bahasa Hindi, yaitu aparajita yang berarti ‘yang tak terkalahkan’. Tanaman ini sekurang-kurangnya disebutkan pada dua kitab utama Ayurveda, yaitu Charaka Samhita dan Sushruta Samhita (Kumar et al., 2016). Peran terpenting telang di dalam Ayurveda adalah sebagai salah satu bahan dalam Medhya Rasayana, yakni campuran herbal yang diperdai berkhias untuk meremajakan otak, menyembuhkan gangguan neurologis dan meningkatkan atau mempertahankan kecerdasan (Lijon et al., 2017). Tidak semua manfaat tersebut telah dibuktikan secara ilmiah, sehingga hanya dapat dipandang sebagai kearifan masa lalu yang dapat dikembangkan sebagai gagasan penelitian.


Sebagaimana telah disampaikan, tulisan ini khusus merangkum dan mengulas manfaat bunga telang
yang sudah mendapatkan bukti ilmiah. Sementara itu, bagian-bagian lain dari telang, yang juga banyak memiliki manfaat, berada di luar cakupan artikel ini.

**Sifat Fungsional dan Nutraseutikal Bunga Telang**

Dunia pengetahuan sudah lama menyadari manfaat produk-produk metabolisme sekunder untuk menopang kesehatan tubuh manusia. Di antara produk metabolisme sekunder itu adalah polifenol. Bunga telang adalah salah satu dari sumber tanaman dengan kadar polifenol relatif tinggi sehingga potensial memberikan manfaat kesehatan bagi manusia (Kamkaen & Wilkinson, 2009; Marpaung et al., 2013; Rabeta & An Nabil, 2013).

Pada bab ini dirangkum berbagai penelitian yang mengungkapkan manfaat ekstrak bunga telang sebagai antioksidan, antidiabetes, anti-obesitas, anti-inflamasi, antimikroorganisme, antikanker, hepatoprotektif, dan beberapa manfaat fungsional lainnya.

**Antioksidan**


Aktivitas antioksidan dalam mengelola stres oksidatif pada sistem biologis berlangsung melalui berbagai mekanisme seperti penangkapan radikal bebas, penghambatan enzim oksidatif, sebagai pengketel ion logam, dan sebagai kofaktor enzim antioksidan (Lakshan et al., 2019). Di antara metode yang umum untuk menguji kemampuan suatu sumber untuk menangkap radikal bebas adalah metode DPPH (2,2-DiPhenyl 1-PicrylHydrazyl), ABTS (2,2′-Azinobis(3-ethylBenzoThiazoline-6-Sulfonate), ORAC (Oxygen radical absorbance capacity), FRAP (Ferric-Reducing Antioxidant Power) dan TEAC (Trolox equivalent antioxidant capacity). Metode lain yang juga dikenal adalah HRSA (Hydroxyl radical scavenging activity), dan SRSA (Superoxide radical scavenging activity). Aktivitas antioksidasi bunga telang yang diekstraksi dengan berbagai prosedur dan pelarut serta diuji melalui berbagai metode telah dilaporkan pada sejumlah penelitian (Tabel 1).

Paling tidak ada tiga cara untuk menakar kemampuan suatu sumber sebagai antioksidan. Cara pertama, adalah mengukur seberapa banyak (dalam %) senyawa radikal yang dinetralkan oleh sumber antioksidan pada konsentrasi tertentu. Cara kedua, cara yang lebih umum dan komparatif, adalah menentukan konsentrasi sumber antioksidan untuk menetralkan 50% senyawa radikal, atau yang biasa dikenal dengan IC₅₀. Cara ketiga adalah dengan menentukan konsentrasi efisien untuk mencapai 50% dari respons maksimum dari suatu sumber atau EC₅₀. Dengan mengetahui IC₅₀ atau EC₅₀ kinerja suatu sumber antioksidan dapat dibandingkan dengan kinerja sumber antioksidan lain atau dengan kinerja antioksidan standar, biasanya adalah vitamin C (asam askorbat). Semakin kecil IC₅₀ atau EC₅₀ semakin efektif kerja suatu sumber sebagai antioksidan.

Sebagian besar peneliti menyebutkan bahwa kemampuan bunga telang untuk mereduski senyawa radikal masih lebih rendah dibandingkan dengan kemampuan vitamin C (Rabeta & An Nabil, 2013; Srichaikul, 2018; Rajamanickam et al., 2015; Chayaratanasin et al., 2015; Phrueksanan et al., 2014). Rajamanickam et al. (2015) melaporkan bahwa IC₅₀ ekstrak metanol bunga telang adalah 95,30 mg/ml, sedangkan vitamin C hanya 70,80 mg/ml. Menurut Phrueksanan et al. (2014) IC₅₀ ekstrak air bunga telang adalah 0,47 mg/ml atau kira-kira 235 kali lebih tidak efektif dibandingkan vitamin C yang
<table>
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<th>Referensi</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>IC₅₀ (µg/ml)</td>
<td>EC₅₀ (µg/ml)</td>
</tr>
<tr>
<td>Air</td>
<td>DPPH</td>
<td>470</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>242</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>84</td>
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</tr>
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<td></td>
<td></td>
<td></td>
<td>0,43</td>
</tr>
<tr>
<td>Air</td>
<td></td>
<td>25</td>
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</tr>
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<td></td>
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<td>50</td>
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<td></td>
<td></td>
<td>125</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>150</td>
<td>507</td>
</tr>
<tr>
<td>Metanol</td>
<td>95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Etanol</td>
<td>4000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kloroform</td>
<td>132</td>
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<td>Etil asetat</td>
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<tr>
<th>Pelarut</th>
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<th>Konsentrasi (µg/ml)</th>
<th>% Penghambatan</th>
<th>IC₅₀ (µg/ml)</th>
<th>EC₅₀ (µg/ml)</th>
<th>Referensi</th>
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<tr>
<td>Air</td>
<td>SRSA</td>
<td>26310</td>
<td></td>
<td></td>
<td></td>
<td>(Chayaratanasin et al., 2015)</td>
</tr>
<tr>
<td>Air</td>
<td>HRSA</td>
<td>19180</td>
<td></td>
<td></td>
<td></td>
<td>(Chayaratanasin et al., 2015)</td>
</tr>
<tr>
<td>Air</td>
<td>ABTS</td>
<td>0,1</td>
<td></td>
<td>4,16</td>
<td></td>
<td>(Srichaikul, 2018)</td>
</tr>
<tr>
<td>Air</td>
<td>ABTS</td>
<td>µM TEAC/g</td>
<td></td>
<td>0,38</td>
<td></td>
<td>(Chayaratanasin et al., 2015)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,33</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>0,78</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Air</td>
<td>FRAP</td>
<td>µg FeSO₄/mg</td>
<td></td>
<td>0,17</td>
<td></td>
<td>(Chayaratanasin et al., 2015)</td>
</tr>
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<td></td>
<td></td>
<td>10,91</td>
<td></td>
<td></td>
<td></td>
<td>(Siti Azima et al., 2017)</td>
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<tr>
<td>Air</td>
<td>FRAP</td>
<td>mM TEAC/mg</td>
<td></td>
<td></td>
<td></td>
<td>(Chayaratanasin et al., 2015)</td>
</tr>
<tr>
<td></td>
<td>TEAC</td>
<td>mg TEAC/mg</td>
<td></td>
<td></td>
<td></td>
<td>(Chayaratanasin et al., 2015)</td>
</tr>
<tr>
<td>Air</td>
<td>ORAC</td>
<td>µM TEAC/g</td>
<td></td>
<td>15,76</td>
<td></td>
<td>(Siti Azima et al., 2017)</td>
</tr>
</tbody>
</table>

Memiliki IC₅₀ 0,002 mg/ml. Menurut (Iamsaard et al., 2014) IC₅₀ ekstrak air bunga telang adalah 84,15 µg/ml, sedangkan IC₅₀ asam ascorbat adalah 5,34 µg/ml. Kontradiktif dengan para peneliti lain, Suganya et al., (2014) menyebutkan bahwa kemampuan ekstrak bunga telang untuk mereduksi senyawa radikal lebih tinggi dibandingkan dengan vitamin C.
Pada penelitian lain dilaporkan bahwa efektivitas bunga telang hanya 10,5% dari efektivitas Trolox untuk menangkap radikal hidroksil dan hanya 2% dari efektivitas Trolox untuk menangkap radikal superoksida. (Chayaratanasin et al., 2019). Uji aktivitas antioksidasi dengan berbagai metode menunjukkan bahwa ekstrak bunga telang memiliki kemampuan yang baik di dalam menangkap berbagai macam radikal bebas, tetapi tergolong sebagai pengkelat logam yang lemah (Chayaratanasin et al., 2015).

Studi terhadap aktivitas antioksidasi 15 jenis bunga menunjukkan bahwa ekstrak bunga telang merupakan salah satu dari bunga yang memiliki aktivitas antioksidasi paling tinggi (Vankar & Srivastava, 2010). Akan tetapi, menurut Siti Azima et al. (2017) aktivitas antioksidasi bunga telang masih lebih rendah dibandingkan dengan aktivitas kulit manggis (Garcinia mangostana), buah Ardisia colorata, dan buah jabangl (Syzygium cumini), baik dengan metode DPPH, ABTS, maupun FRAP. Berdasarkan metode ORAC aktivitas antioksidan bunga telang lebih baik dibandingkan dengan buah Ardisia colorata dan buah jabangl. Menurut (Lakshmeesh, 2019) bunga mawar lebih efektif dibandingkan dengan bunga telang sebagai antioksidan.

Ekstrak air bunga telang memiliki aktivitas antioksidasi yang lebih baik dibandingkan dengan ekstrak pelarut organik (Kamkaen & Wilkinson, 2009; Rabeta & An Nabil, 2013). Sementara itu, ekstrak metanol menghambat oksidasi dengan lebih baik dibandingkan dengan ekstrak etil asetat dan ekstrak kloroform (Rajamanickam et al., 2015). Hasil-hasil ini mengindikasikan bahwa fraksi hidrofilik (polar) bunga telang lebih berperan sebagai antioksidan daripada fraksi lipofilik atau nonpolaranya.

Potensi ekstrak bunga telang kemudian dipelajari lebih lanjut untuk melihat efektifitasnya di dalam melindungi sel dari kerusakan akibat oksidasi. Satu penelitian menunjukkan bahwa ekstrak bunga telang melindungi eritrosit anjing dari hemolisosis dan kerusakan oksidatif yang disebabkan oleh 2,20 – azobis – 2 – metil - propanimidamide dihydrochloride (AAPH) (Phruksanana et al., 2014). Ekstrak bunga telang juga efektif melindungi sel-sel kulit dari tekanan oksidatif yang diinduksi oleh hidrogen peroksida dan sinar ultraviolet, yang membuatnya potensial sebagai kosmetika untuk memperlambat kulit keriput (Zakaria et al., 2018). Dalam studi lain sifat antioksidan dalam ekstrak bunga telang memfasilitasi produksi nanopartikel magnesium oksida, bahan yang semakin banyak digunakan untuk aplikasi biomedis (Sushma et al. 2015).

**Antidiabetes**

Diabetes Mellitus (DM) merupakan gangguan metabolik yang ditandai oleh terjadinya hiperglikemia (gula darah tinggi), dislipidemia (gangguan metabolism lipoprotein), dan metabolism protein abnormal akibat terganggunya sekresi dan atau kerja insulin. Prosedur yang paling umum untuk menguji potensi antidiabetes suatu bahan adalah dengan mengukur efek hipoglikemia atau antihiperglikimia (menurunkan gula darah) bahan tersebut pada hewan percobaan, biasanya adalah tikus yang dibuat mengalami diabetes dengan cara diinduksi alloxan. Alloxan menyebabkan penurunan ekksresi insulin secara drastis akibat kerusakan sel-β pulau Langerhans pada pankreas, sehingga menginduksi terjadinya hiperglikemia.

Efek hipoglikemia ekstrak bunga telang telah dibuktikan melalui beberapa penelitian (Daisy et al., 2009; Rajamanickam et al., 2015; Chusak et al., 2018). Pemberian ekstrak air bunga telang secara oral (400 mg/kg berat badan) kepada tikus percobaan menurunkan glukosa serum dan glikosilasi hemoglobin, serta meningkatkan insulin serum, glikogen otot hati dan tulang (Daisy et al., 2009). Pemberian ekstrak metanol, etil asetat, atau kloroform sebanyak 300 mg/kg berat badan menunjukkan aktivitas hipoglikemia pada tikus albino yang lebih efektif daripada obat diabetes komersial glibencamide (10 mg/kg) (Rajamanickam et al., 2015). Ekstrak kloroform bekerja lebih baik dibandingkan dengan ekstrak etil asetat dan methanol yang mengindikasikan bahwa aktivitas hipoglikemia lebih dikontribusikan oleh komponen bioaktif non-polar. Aktivitas antihiperglikemia ekstrak bunga telang telah pula diamati pada 15 pria sehat berusia rata-rata 22,53 tahun dengan indeks massa tubuh rata-rata 21,57.
kg/m² yang diberi diet minuman yang mengandung 50 g sukrosa. Setelah 30 menit konsumsi, subjek yang minum minuman mengandung sukrosa bersama dengan ekstrak bunga telang (2 g/400 ml air atau setara dengan 2,16 mg delfinidin 3-glukosida) memiliki kadar glukosa plasma dan insulin postprandial yang lebih rendah (Chusak et al., 2018). Selain itu, konsumsi ekstrak bunga telang juga meningkatkan kapasitas antioksidan plasma dan menurunkan kadar malondialdehida (MDA) yang merupakan penanda stress oksidatif (Chusak et al., 2018).

Mekanisme hipoglikemia ekstrak bunga telang diperkirakan melalui peningkatan sekresi insulin sebagaiana cara kerja glibencamide (Rajamanickam et al., 2015) yang ditandai dengan meningkatnya insulin serum dan kadar glikogen (Daisy et al., 2009).

Aktivitas antidiabetes suatu komponen aktif dapat pula melalui penghambatan pembentukan produk akhir glikasi lanjut (advanced glycation end products - AGEs). Ekstrak bunga telang pada konsentrasi 0,25-1,00 mg/ml dilaporkan secara signifikan menghambat pembentukan AGE, serta mengurangi kadar fruktosamin dan oksidasi protein dengan mengurangi kandungan karbonil protein dan mencegah penipisan tiol bebas (Chayaratanasin et al., 2019).

Keadaan hiperglikemia dapat pula dihadang melalui penghambatan kerja enzim-enzim yang berhubungan dengan produksi glukosa dalam tubuh. Mukherjee et al. (2008) menyebutkan bahwa ekstrak etanol bunga telang menghambat produksi glukosa melalui penghambatan enzim β-galactosidase dan β-glucosidase, tetapi tak ada penghambatan terhadap aktivitas enzim β-d-fructosidase. Daisy et al. (2009) melaporkan bahwa ekstrak bunga telang menghambat aktivitas enzim glukoneogenik, glukosa-6-fosfatase, dan sebaliknya meningkatkan aktivitas enzim glukokinase. Glukokinase adalah enzim yang bertanggungjawab untuk mengubah glukosa menjadi glukosa 6-fosfat yang merupakan langkah pertama untuk membatasi metabolisme glukosa. Potensi ekstrak bunga telang untuk menghambat enzim α-amilase pankreas dan α-glucosidase usus besar telah pula dibuktikan (Adisakwattana et al., 2012). Penelitian yang lebih baru membuktikan bahwa 1% dan 2% (b/v) ekstrak bunga telang menghambat aktivitas enzim α-amilase pankreas dengan substrat zat pati yang berasal dari tepung kentang, singkong, beras, jagung, gandum, dan beras ketan sehingga diusulkan untuk digunakan sebagai bahan untuk mengurangi indeks glikemik berbagai jenis tepung (Chusak et al., 2018). Kembapan ekstrak air bunga telang untuk menghambat kerja enzim α-amilase secara in vitro juga dilaporkan pada penelitian sebelumnya (Chu et al., 2017).

Upaya untuk meningkatkan efek hipoglikemia ekstrak bunga telang dengan cara mengombinasikannya dengan ekstrak buah lain menunjukkan hasil yang positif (Adisakwattana et al., 2012; Borikar et al., 2018). Kombinasi ekstrak bunga telang dengan rosela meningkatkan aktivitas penghambatan enzim α-amilase, sedangkan kombinasi ekstrak bunga telang dengan mulberi meningkatkan aktivitas penghambatan enzim α-glucosidase (Adisakwattana et al., 2012). Kombinasi bunga telang dan buah delima buah delima (Punica gratum) menghasilkan aktivitas hipoglikemik yang setara dengan obat diabetes metformin (Borikar et al., 2018).

**Antiobesitas, Antihiperlipidemik dan Regulasi Kolesterol**

Obesitas terkait dengan pembentukan jaringan lemak. Oleh karena itu potensi suatu bahan aktif sebagai antiobesitas seringkali dipelajari melalui kemampuannya menghambat adipogenesis (pembentukan jaringan lemak) pada preadiposit 3T3-L1 (lini sel yang diisolasi dari jaringan embrio tikus Swiss albino). Aktivitas anti adipogenesis ekstrak bunga telang baru-baru ini dilaporkan oleh Chayaratanasin et al. (2019).

Sementara itu, dalam rangkaian kajian terhadap aktivitas ekstrak bunga telang melawan diabetes pada tikus percobaan, peran bunga telang untuk menurunkan trigliserida dan total kolesterol darah dan meningkatkan kadar kolesterol-HDL telah pula dibuktikan (Daisy et al., 2009; Suganya et al., 2014; Rajamanickam et al., 2015).
Antikanker

Sekurang-kurangnya terdapat empat mekanisme dari suatu komponen zat aktif untuk melawan kanker: aktivitas antiproliferasi (mencegah atau memperlambat penyebaran sel kanker, penghambatan angiogenesis (pembentukan pembuluh darah baru), induksi apoptosis (sel kanker melakukan bunuh diri), pencegahan metastasis.

Aktivitas anti-proliferasi ekstrak bunga telang terhadap enam jenis lini sel kanker diamati oleh Neda et al. (2013). Peneliti ini melaporkan bahwa ekstrak air bunga telang potensial menghambat lini sel kanker payudara MCF-7 dan tidak efektif menghambat lini sel kanker payudara MDA-MB-231, lini sel kanker ovarium (Caov-3), lini sel kanker serviks (Hela), lini sel kanker hati (HepG2) dan lini sel kanker kelamin pria (Hs27). Analisis GC-MS (Gas Chromatogram-Mass spectrometry) menunjukkan bahwa dua komponen aktif pada ekstrak air bunga telang adalah mome inositol (38,7%) dan pentanal (14,3%) (Neda et al., 2013).

Efektivitas ekstrak bunga telang untuk menghambat MCF-7 (IC$_{50}$ = 1.14 mg/ml) dilaporkan pula oleh Akter et al. (2014). Akan tetapi, tak sejalan dengan Neda et al. (2013), Akter et al. (2014) melaporkan bahwa ekstrak bunga telang sangat efektif menghambat pertumbuhan sel MDA-MB-231 (IC$_{50}$ = 0.11 mg/ml).

Aktivitas anti-proliferatif ekstrak lipofilik dan hidrofilik bunga telang terhadap lini sel kanker laring (Hep-2: human epithelial type 2) dilaporkan oleh (Shen et al., 2016) dengan ekstrak hidrofilik menunjukkan efektivitas yang lebih baik dibandingkan dengan ekstrak lipofilik. Penelitian ini membawa kepada satu perkiraan bahwa fraksi hidrofilik pada bunga telang berperan lebih efektif sebagai antikanker dibandingkan dengan fraksi lipofiliknya.

Efek sitotoksik in vitro dari ekstrak petroleum eter dan etanol bunga telang menggunakan metode trypan blue exclusion menunjukkan bahwa 500 mg/ml ekstrak petroleum eter dan metanol membunuh masing-masing 100% dan 80% lini sel DLA (Dalton’s lymphoma ascites) (Shyam Kumar & Ishwar Bhat, 2011).

Aktivitas antikanker ekstrak metanol bunga telang melalui jalur induksi apoptosis dilaporkan terjadi pada lini sel kanker payudara MCF-7 (Shivapakasha et al., 2015). Terjadinya induksi apoptosis ditandai dengan fragmentasi DNA dan aktivasi enzim Caspase-3.

Angiogenesis adalah pembentukan pembuluh darah baru yang dilakukan oleh sel kanker untuk memperlancar pasokan makanan bagi pertumbuhan sel kanker. Angiogenesis juga memainkan peran penting dalam transisi tumor dari keadaan tak aktif ke stadium ganas. Vascular endothelial growth factor (VEGF) adalah protein yang memegang peran kunci di dalam angiogenesis. Ekstrak metanol bunga telang dilaporkan memiliki aktivitas menekan angiogenesis pada lini sel EAC (Ehrlich ascites carcinoma) dengan cara meregulasi sekresi VEGF. Ekstrak metanol bunga telang juga terlihat menekan aktivitas HIF-1α (Hypoxia Inducible Factor-1α) yang diperkirakan dapat menjadi satu pendekatan baru dalam penghambatan pertumbuhan sel kanker (Balaji et al., 2016).

Antiinflamasi dan Analgesik

Inflamasi atau perdarahan adalah upaya perlindungan tubuh yang bertujuan untuk menghilangkan ransangan berbahaya, termasuk sel-sel yang rusak, iritasi, atau patogen dan memulai proses penyembuhan. Antiinflamasi adalah karakteristik yang dimiliki oleh suatu zat atau komponen untuk mengurangi perdarahan atau peradangan. Bahan antiinflamasi memiliki kemampuan analgesik yang memengaruhi sistem saraf untuk menghambat sinyal nyeri ke otak.

Efek antiinflamasi dan analgesik ekstrak petroleum eter bunga telang (masing-masing dengan kadar 200 mg/kg berat badan dan 400 mg/kg berat badan) pada tikus percobaan dilaporkan oleh Shyam kumar & Ishwar (2012). Sekalipun demikian, efektifitasnya masih jauh lebih rendah dibandingkan dengan obat antiinflamasi dan analgesik komersial (diclofenac sodium dan pentazocine).

Aktivitas inflamasi secara in vitro (penghambatan denaturasi albumin) dari ekstrak etanol bunga telang dipelajari Suganya et al. (2014) pada...
berbagai konsentrasi. Hasilnya menunjukkan bahwa kinerja ekstrak bunga telang setara dengan kinerja aspirin (Suganya et al., 2014).

Efek antiinflamasi ekstrak bunga telang pada peradangan yang diinduksi oleh lipopolisakarida pada lini sel makrofag RAW 264.7 dilaporkan oleh Nair et al. (2015). Hasil ini menunjukkan potensi bunga telang sebagai bahan nutrasetikal untuk perlindungan terhadap penyakit peradangan kronis dengan menekan produksi mediator pro-inflamasi yang berlebihan dari sel makrofag (Nair et al., 2015). Sebagai kompleks dengan sumber antosianin lain, ekstrak juga menunjukkan aktivitas antiinflamasi yang potensial (Priprem et al., 2015; Intuyod et al., 2014)

**Antiasma**

 Salah satu khasiat bunga telang yang dipercaya di dalam pengobatan tradisional India adalah untuk menyembuhkan asma dan meredakan batuk. Asma merupakan gangguan inflamasi kronik pada saluran pernapasan yang dapat menyebabkan penderita mengalami batuk dan sesak napas. Berbagai studi telah dilakukan untuk mengkonfirmasi kinerja bunga telang sebagai antiasma dan pereda batuk (Singh et al., 2018). Rangkaian studi itu meliputi aplikasi ekstrak bunga telang dosis tinggi (100, 200, dan 400 mg/kg berat badan hewan percobaan) sebagai antiasma akut dan kronis, meredakan batuk yang diinduksi sulfur dioksida dan asam sitrat, serta aktivitas anti-inflamasi pada tikus yang diinduksi karagenan dan asam sitrat. Rangkaian studi tersebut menghasilkan satu kesimpulan bahwa ekstrak bunga telang yang terstandar berpotensi sebagai terapi alternatif dalam penanganan asma yang diinduksi oleh alergi (Singh et al., 2018).

**Antimikroorganisme**

Bunga telang yang diekstraksi menggunakan berbagai pelarut menunjukkan rentang aktivitas antimikroorganisme yang luas meliputi bakteri gram positif, bakteri gram negatif maupun fungi (Tabel 2). Di antara aktivitas yang perlu digarisbawahi adalah ekstrak bunga telang menghambat pertumbuhan tiga bakteri patogen yang paling banyak ditemukan pada permukaan tanah, yaitu Bacillus subtilis, Staphylococcus aureus and Escherichia coli (Kamilla et al., 2009; Uma et al., 2009; Pratap et al., 2012; Mahmad et al., 2018). Ekstrak bunga telang juga menghambat pertumbuhan beberapa bakteri patogen enzim extended-spectrum beta-lactamase (ESBL) yaitu E. coli, Enteropathogenic E. coli (EPEC), Enterotoxigenic E. coli (ETEC), Klebsiella pneumoniae dan Pseudomonas aeruginosa (Kamilla et al., 2009; Uma et al., 2009; Pratap et al., 2012). ESBL adalah enzim yang menyebabkan bakteri tahan terhadap berbagai macam antibiotik seperti penisilin dan sefalosporin. Ekstrak bunga telang juga dilaporkan menghambat pertumbuhan tiga bakteri penyebab kerusakan gigi, yaitu Streptococcus mutans, Lactobacillus casei, dan Staphylococcus aureus (Pratap et al., 2012). Penelitian Kamilla et al. (2009) menunjukkan bahwa ekstrak methanol bunga telang menghambat pertumbuhan bakteri Salmonella typhi, sementara Uma et al. (2009) melaporkan bahwa ekstrak methanol, kloroform dan air bunga telang tidak dapat menghambat pertumbuhan bakteri Salmonella typhimurium dan S. enteritis.


**Hepatoprotektif**

Aktivitas hepatoprotetif dievaluasi dengan memantau kadar enzim aspartat aminotransferase dan alanin aminotransferase, serta kadar bilirubin dan glutation melalui analisis hispatologis. Hasil percobaan menunjukkan bahwa tikus yang diberi ekstrak bunga telang (200 mg/kg) mengalami penurunan kadar keempat senyawa indikator kerusakan hati.

Tabel 2. Aktivitas antimikroba pada bunga telang

<table>
<thead>
<tr>
<th>Mikroorganisme</th>
<th>Pelarut</th>
<th>Konsentrasi (mg/ml)</th>
<th>Zona Hambatan (mm)</th>
<th>Pustaka</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bakteri Gram Positif</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>Metanol</td>
<td>100</td>
<td>14±1</td>
<td>Kamilla et al., 2009</td>
</tr>
<tr>
<td></td>
<td>Etanol</td>
<td>50</td>
<td>14,5 ± 2,1</td>
<td>Leong et al., 2017</td>
</tr>
<tr>
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<td>100</td>
<td>2,7±1,1</td>
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</tr>
<tr>
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<td>50</td>
<td>15,8 ± 1,7</td>
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</tr>
<tr>
<td></td>
<td>Etanol</td>
<td>Tds</td>
<td>10,0 ± 0,3</td>
<td>Mahmad et al., 2018</td>
</tr>
<tr>
<td>Bacillus thuringiensis</td>
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<td>100</td>
<td>15,7±0,6</td>
<td>Kamilla et al., 2009</td>
</tr>
<tr>
<td></td>
<td>Etanol</td>
<td>50</td>
<td>13 ± 1</td>
<td>Kamilla et al., 2009</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>50</td>
<td>8</td>
<td>Pratap et al., 2012</td>
</tr>
<tr>
<td></td>
<td>Etanol</td>
<td>Tds</td>
<td>7,0 ± 0,7</td>
<td>Mahmad et al., 2018</td>
</tr>
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<td>100</td>
<td>13±1</td>
<td>Kamilla et al., 2009</td>
</tr>
<tr>
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<td>Metanol</td>
<td>100</td>
<td>12 ± 1</td>
<td>Kamilla et al., 2009</td>
</tr>
<tr>
<td>Streptococcus mutans</td>
<td>Air</td>
<td>50</td>
<td>7</td>
<td>Pratap et al., 2012</td>
</tr>
<tr>
<td><strong>Bakteri Gram Negatif</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>Escherichia coli</td>
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<td>100</td>
<td>13,3 ± 0,6</td>
<td>Kamilla et al., 2009</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>4*</td>
<td>12</td>
<td>Uma et al., 2009</td>
</tr>
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<td></td>
<td>Kloroform</td>
<td>4*</td>
<td>14</td>
<td>Uma et al., 2009</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>Metanol</td>
<td>100</td>
<td>12,7 ± 0,6</td>
<td>Kamilla et al., 2009</td>
</tr>
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<td></td>
<td>Air</td>
<td>4*</td>
<td>12</td>
<td>Uma et al., 2009</td>
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<td>Metanol</td>
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<td>12,0 ± 0,4</td>
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<td>Air</td>
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<td>12</td>
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<td>Kloroform</td>
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</tr>
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<td>Salmonella typhi</td>
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<td>14,0 ± 1,1</td>
<td>Leong et al., 2017</td>
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<tr>
<td>Herbaspirillum spp</td>
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<td>11,3 ± 2,3</td>
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<td>Streptococcus mutans</td>
<td>Air</td>
<td>50</td>
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<td>Air</td>
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<td><strong>Antifungi</strong></td>
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</tr>
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<td>Mahmad et al., 2018</td>
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<td>Trichoderma sp.</td>
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<td>Tds</td>
<td>8,0 ± 0,5</td>
<td>Mahmad et al., 2018</td>
</tr>
</tbody>
</table>

tds = tidak disebutkan; *konsentrasi dalam mg/disc
Komponen Bioaktif Pada Bunga Telang

Kinerja farmakologis bunga telang merupakan kontribusi dari berbagai komponen aktif, baik yang berasal dari metabolism primer maupun sekunder, baik yang bersifat hidrofilik maupun lipofilik. Pada bab ini dibahas komponen aktif pada bunga telang yang telah berhasil diidentifikasi hingga saat ini.

Komponen metabolit primer utama pada bunga telang adalah lemak, yaitu sebanyak 32,9% per berat kering. Berikutnya adalah karbohidrat (29,3%) dan serat kasar (27,6%). Sementara itu, protein dijumpai dalam kadar yang relatif kecil (4,2%) (Neda et al., 2013). Komponen bioaktif pada bunga telang yang diperkirakan memiliki manfaat fungsional berasal dari berbagai kelompok senyawa fitokimia, yaitu fenol (flavonoid, asam fenolat, tanin, dan antrakuinon), terpenoid (triterpenoid, saponin tokoferol, fitosterol), dan alkaloid.

Komponen bioaktif lipofilik terdapat dalam jumlah lebih banyak dibandingkan dengan komponen hidrofilik, masing-masing sebesar 27,67 dan 11,08 mg/100 g bunga segar (Shen et al., 2016). Pada fraksi lipofilik, yang paling banyak adalah kelompok fitosterol dan asam lemak. Sementara itu pada fraksi hidrofilik yang terbanyak adalah antosianin dan flavonol glicosida (Kazuma et al., 2003; Shen et al., 2016).

Menurut sumber lain kandungan total senyawa fenol pada bunga telang berkisar antara 53-460 mg ekuivalen asam galat per gram ekstrak kering (Adisakwattana et al., 2012; Chayaratanasin et al., 2015; Singh et al., 2018). Senyawa-senyawa fenol tersebut terdiri dari flavonoid, asam fenolat dan tanin. Komponen bioaktif bukan fenol yang telah diidentifikasi pada bunga telang adalah kelompok senyawa fitosterol, terpena, gula alkohol, alkil aldehida dan peptida.

Flavonoid

Satu gram ekstrak kering bunga telang mengandung flavonoid rata-rata 11.2 mg ekuivalen katekin (Chayaratanasin et al., 2015). Flavonoid 25,8 mg setara kuersetin per gram ekstrak (Singh et al., 2018). Komponen flavonoid pada bunga telang adalah flavonol, antosianidin, flavanol, dan flavon (Gambar 2).

Flavonol glicosida

Di dalam bunga telang flavonol dijumpai dalam bentuk glikonnya, yaitu flavonol glicosida, yang terdiri dari kaempferol 3-glukosida (kaempferol 3-(2-rhamnosilrutinosida), kaempferol 3-neohesperidosida, kaempferol 3-(2-rhamnosil-6-malonil) glukosida, kaempferol 3-rutinosida), kuersetin 3-glukosida (kuersetin 3-(2-rhamnosilrutinosida), kuersetin 3-neohesperidosida, kuersetin 3-rutinosida, kuersetin 3-glucosida) dan mirisetin 3-glicosida (mirisetin 3-(2-rhamnosilrutinosida)) (Kazuma et al., 2003).

Flavonol glicosida merupakan flavonoid yang paling banyak dijumpai pada bunga telang, dan yang paling utama adalah kaempferol 3-glikosida yang kandungannya sekitar 87% total flavonol glicosida (Kazuma et al., 2003).

Kaempferol, kuersetin, dan mirisetin merupakan komponen bioaktif yang mudah diperoleh di berbagai jenis tanaman. Senyawa-senyawa flavonol ini memiliki manfaat kesehatan yang luas. Kuersetin dikenal sebagai antioksidan diet yang paling menonjol (Boots et al., 2008).

Antosianin

Sama dengan flavonol, antosianidin dalam bunga telang dijumpai dalam bentuk glikonnya, antosianin. Karakteristik bunga yang paling menonjol secara visual adalah warnanya yang biru pekat yang disebabkan oleh antosianin yang dikandungnya. Sekalipun demikian, antosianin bukanlah flavonoid yang paling banyak di dalam bunga telang. Fraksi antosianin hanya sekitar 27% dari total flavonoid dalam bunga telang (Kazuma et al., 2003).

Antosianin bunga telang merupakan antosianin terpoliasilasi (memiliki lebih dari dua gugus asil) dengan delfinidin sebagai aglikonnya. Antosianin terpoliasilasi memiliki kestabilan lebih tinggi dibandingkan dengan jenis antosianin yang tak memiliki gugus asil.
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Antosianin secara umum dikenal sebagai kelompok pigmen larut air yang memiliki manfaat fungsional yang luas. Semua antosianin adalah antioksidan dan merupakan anggota keluarga flavonoid dengan aktivitas antioksidan paling tinggi. Aktivitas antioksidan antosianin adalah karena kemampuannya menyumbang hidrogen kepada radikal dan membantu mengakhiri reaksi radikal berantai (Iversen, 1999). Aktivitas antioksidan antosianin yang satu berbeda dengan antosianin yang lain tergantung kepada perubahan筢 pada jumlah dan susunan gugus hidroksil dan gula terkonjugasi.

Selain itu, antosianin juga menunjukkan sifat anti-virus, antiinflamasi, antioksidan, anti-alergi, dan antimikroba, antikanker, anti-arteri aterosklerosis, anti-hipertensi, mencegah diabetes, melindungi sistem kardiovaskular dari kerusakan dan banyak manfaat kesehatan lainnya (Ghosh & Konishi, 2007; Khoo et al., 2017). Studi klinis telah menunjukkan efek menguntungkan antosianin pada manusia seperti meningkatkan kadar kolesterol HDL dan menurunkan kadar kolesterol LDL pada subyek dislipidemik, mengurangi risiko infark miokard pada wanita muda dan setengah bayi, dan mengurangi risiko penyakit kardiovaskuler (Intuyod et al., 2014).

Akan tetapi masih sangat sedikit studi terkait efek fungsional dari antosianin seri ternatin yang khas dimiliki oleh bunga telang. Hingga saat ini baru ada dua laporan yang berhasil dilacak terkait efek fungsional ternate. Pertama, aktivitas ternatin D1 untuk menghambat agregasi platelet secara in vitro (Kshirsagar et al., 2015). Kedua, ternatin menghambat translokasi NF-κB nuklir, ekspresi protein iNOS, dan produksi NO (Nair et al., 2015).

**Flavon dan flavanol**

Dalam riset mereka untuk memahami bagaimana bunga telang memiliki kemampuan menghambat angiogenesis pada sel kanker, Balaji et al. (2016) mengidentifikasi keberadaan empat senyawa flavon di dalam ekstrak methanol bunga telang. *Scutellarin* adalah yang paling banyak (36,9%), diikuti oleh *baicalein* (12,6%), luteolin (9,3%) dan apigenin (6,3%). Keempat senyawa flavon tersebut diketahui memiliki efek antikanker pada sejumlah sel kanker melalui beberapa mekanisme. Efek farmakologis termasuk sebagai zat antikanker dari *scutellarin*, apigenin, *baicalein* dan luteolin telah dibahas mendalam pada beberapa referensi (Wang & Ma, 2018; Salehi et al., 2019; Liu et al., 2016; Lin et al., 2008)

Satu-satunya senyawa flavanol yang telah dipastikan ada dalam bunga telang adalah epikatekin (Siti Azima et al., 2017). Epikatekin merupakan senyawa polifenol yang banyak dijumpai pada coklat dan teh. Bersama dengan flavon, katekin merupakan flavonoid yang paling tangguh melindungi tubuh dari spesies oksigen reaktif (Tapas et al., 2008).

**Asam Fenolat**

Asam fenolat terbagi ke dalam dua kelompok senyawa, yaitu asam hidroksisinamat dan asam hidroksibenzoat (Gambar 4). Asam hidroksisinamat lebih banyak dijumpai di alam daripada asam hidroksibenzoat (Kumar et al., 2014). Para peneliti menunjukkan hasil penelitian yang tidak selalu sejalan dengan jenis asam fenolat apa saja yang terdapat pada bunga telang, hal ini disebabkan oleh perbedaan pelarut untuk ekstraksi dan prosedur isolasi. Secara keseluruhan, asam hidroksisinamat yang dijumpai pada bunga telang adalah asam klorogenat, asam galat, asam p-kumarat, asam kafeat, asam ferulat, sedangkan asam hidroksibenzoat pada bunga telang adalah asam protokatekuat, asam p-hidroksibenzoat, asam siringat dan asam vanilat (Kaisoon et al., 2011; Siti Azima et al., 2017; Pengkumsri et al., 2019). Menurut Siti Azima et al. (2017) urutan asam fenolat yang terdapat paling banyak adalah asam protokatekuat (72 mg/100 g), asam galat (67 mg/100 g) dan asam klorogenat (54 mg/100 g).
Asam fenolat adalah salah satu fitokimia fenol yang paling penting (Dillard & German, 2000). Sama dengan antosianin, semua asam fenolat adalah antioksidan. Asam fenolat memiliki sifat antioksidan karena potensi redoksnya yang tinggi, yang memungkinkan mereka bertindak sebagai agen pereduksi dan mengikat oksigen singlet (Kumar et al., 2014). Di antara keluarga asam fenolat, asam klorogenat adalah yang paling berlimpah di alam sekaligus antioksidan yang paling kuat (Larson, 1988).

Satu catatan menarik, asam protokatekuat dan asam klorogenat merupakan produk akhir dari degradasi antosianin. Oleh karena itu, ketika antosianin terdegradasi menjadi kedua asam fenolat sehingga kehilangan warna secara permanen, aktivitas antioksidannya tetap bertahan.

**Terpenoid**

Hingga saat ini kelompok senyawa terpenoid yang berhasil ditemukan pada bunga telang adalah triterpenoid (yang kemudian diidentifikasi sebagai tarakserol), fitosterol, dan tokoferol (Gambar 5) (Shyam Kumar & Ishwar Bhat, 2011; Shyam Kumar & Ishwar Bhat, 2012; Suganya et al., 2014; Shen et al., 2016; Singh et al., 2018; Zakaria et al., 2018)

Secara umum triterpenoid memiliki aktivitas inflamasi, sehingga diperkirakan efek antiinflamasi dan analgesik bunga telang dikontribusikan oleh tarakserol (Shyam Kumar & Ishwar Bhat, 2012).

Di dalam 100 g bunga segar terdapat rata-rata 15,91 mg fitosterol dengan komposisi kampesterol 1,24 mg, stigmasterol 6,70 mg, β-Sitosterol 6,77 mg, dan sitostanol 1,20 mg (Shen et al., 2016). 1,24, 76,70, 6,77, 1,20 mg/100 g. Fitosterol dipastikan memiliki fungsi hipokolesterolernik dan mengurangi risiko hiperplasia jinak, penyakit kardiovaskular, perkembangan kanker usus dan payudara, serta efek imunologis pada makrofag. Dari keempat fitosterol, β-sitosterol adalah yang paling efektif dalam menghambat pertumbuhan sel kanker melalui aktivasi enzim tertentu, yang pada gilirannya menginduksi apoptosis seluler. β-sitosterol dan kampesterol dapat mengurangi perkembangan kanker melalui penghambatan pembentukan senyawa karsinogen dalam metabolisme biologis (Shen et al., 2016).

Terdapat dua senyawa tokoferol pada bunga telang, yaitu α-tokoferol dan γ-tokoferol, masing-masing sebanyak 0,20 dan 0,24 mg per 100 g bunga segar (Shen et al., 2016). Tokoferol telah dibuktikan melindungi membrane sel terhadap radikal lipida reaktif, mencegah aterosklerosis dan karsinogenesis (Shen et al., 2016).

**Alkaloid**

Keberadaan alkaloid di dalam bunga telang secara kualitatif disebutkan pada beberapa referensi (Uma et al., 2009; Manjula et al., 2013; Suganya et al., 2014; Singh et al., 2018). Akan tetapi tidak ada catatan yang berhasil mengungkapkan identitas senyawa alkaloid tersebut.
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Peptida: Siklotida


Komponen lain

Komponen-komponen lain yang juga ditemukan pada bunga telang adalah asam lemak palmitat, stearat, petroselinat, linolet, arakhidat, behenat dan fitanat (Shen et al., 2016), mome-inositol dan pentanal (Neda et al., 2013). Asam fitanat memiliki peran di dalam mengatur trigliserida atau kolesterol dalam otot rangka (Shen et al., 2016).
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Marpaung, A.M.

## Tantangan Bunga Telang Sebagai Pangan Fungsional dan Nutrasetikal

Secara umum, masih diperlukan perjalanan riset yang relatif panjang untuk sampai pada aplikasi komersial bunga telang sebagai pangan fungsional atau nutrasetikal. Hal ini mengingat penelitian yang telah dilakukan hingga saat ini masih sampai pada tahap in vitro (misalkan pada sel kanker untuk menguji aktivitas antikanter) atau in vivo (pada hewan percobaan untuk menguji aktivitas antidiabetes). Akan tetapi, aplikasi bunga telang sebagai minuman pengontrol gula darah dapat dikatakan sudah dekat dengan aplikasi komersial.

## Pengontrol Gula Darah

Penelitian Chusak et al. (2018) menunjukkan bahwa bunga telang dapat diolah menjadi minuman pengatur gula darah melalui proses yang relatif sederhana, yakni dengan maserasi atau perendaman dalam air sehingga mencapai kepekatan yang setara dengan 2,16 mg delfinidin 3-glukosida per sajian. Konsentrasi ini dapat diperoleh dengan merendam 10 hingga 15 helai bunga telang di dalam 250 ml air panas selama 15 hingga 30 menit.

Salah satu keunggulan bunga telang, sekaligus juga kekurangannya adalah warnanya yang biru-ungu atau biru pekat pada pH 4-6. Sementara, sebagian besar sumber antosianin lainnya tak berwarna atau memiliki warna merah pada pH tersebut. Warna biru bunga telang ini dapat menjadi daya tarik sensoris, tetapi dapat pula menurunkan nilai sensoris. Survei awal yang dilakukan terhadap lebih dari 400 responden menunjukkan adanya kecenderungan konsumen untuk tidak menyukai warna biru yang terlalu pekat. Kabar baiknya adalah minuman yang efektif menunjukkan aktivitas fungsional. Sebagai contoh sebagai antidiabetes. Penelitian Daisy et al. (2009) menunjukkan bahwa bunga telang menunjukkan aktivitas antidiabetes yang signifikan pada tikus percobaan dengan jumlah asupan 400 mg ekstrak kering/kg berat badan tikus. Ekstrak kering ini diperoleh melalui perebusan 100 g bunga kering (kira-kira setara dengan 1000 g bunga segar) dalam 1 liter air, yang dilanjutkan dengan filtrasi, evaporation, dan pengeringan. Konsentrasi setinggi ini menghasilkan warna biru yang sangat pekat yang mungkin sulit diterima secara inderawi. Sebagai perbandingan, minuman bunga telang pada Gambar 5 diperoleh melalui maserasi 10 helai bunga telang sekitar 3 g dalam 250 ml air.

## Kombinasi Dengan Sumber Antosianin Lain

Beberapa penelitian menunjukkan bahwa aplikasi bunga telang dalam bentuk campuran dengan rosela, mulberi dan delima dapat meningkatkan aktivitas fungsionalnya (Adisakwattana et al., 2012; Borikar et al., 2018). Hasil penelitian ini membuka peluang penelitian untuk mempelajari efek kombinasi ekstrak bunga telang dengan ekstrak sumber antosianin lain.

Sebagaimana telah diketahui, bunga telang menampilkan warna yang pekat pada pH produk pangan, sedangkan kebanyakan sumber antosianin lain hampir tak berwarna. Sifat yang bertolak belakang ini dapat dimanfaatkan untuk menghasilkan produk berkadar antosianin lebih tinggi, dengan warna yang tak terlalu pekat sehingga atraktif secara inderawi.

Kadar antosianin yang lebih tinggi mungkin diperlukan untuk mencapai konsentrasi yang efektif menunjukkan aktivitas fungsional. Sebagai contoh sebagai antidiabetes. Penelitian Daisy et al. (2009) menunjukkan bahwa bunga telang menunjukkan aktivitas antidiabetes yang signifikan pada tikus percobaan dengan jumlah asupan 400 mg ekstrak kering/kg berat badan tikus. Ekstrak kering ini diperoleh melalui perebusan 100 g bunga kering (kira-kira setara dengan 1000 g bunga segar) dalam 1 liter air, yang dilanjutkan dengan filtrasi, evaporation, dan pengeringan. Konsentrasi setinggi ini menghasilkan warna biru yang sangat pekat yang mungkin sulit diterima secara inderawi. Sebagai perbandingan, minuman bunga telang pada Gambar 5 diperoleh melalui maserasi 10 helai bunga telang sekitar 3 g dalam 250 ml air.
Fokus Pada Ternatin dan Siklotida

Sekalipun sudah banyak komponen bioaktif yang telah berhasil diidentifikasi dalam bunga telang, masih belum jelas komponen mana yang mengambil peran utama pada aktivitas fungsional tertentu. Sebagai tambahan, sebagian besar komponen bioaktif bunga telang dijumpai pada banyak sumber tanaman lain, sehingga menjadikannya kurang istimewa. Kecuali ternatin dan siklotida. Oleh karena itu adalah beralasan untuk memberikan fokus kepada penelitian manfaat fungsional ternatin dan siklotida.


Siklotida menarik perhatian, karena membawa harapan baru bagi komponen antikanker yang stabil terhadap panas, bahan kimia, dan enzim. Bunga telang diketahui sebagai salah satu sumber siklotida yang paling kaya (Nguyen et al., 2016). Kajian terhadap siklotida telang telah menunjukkan aktivitas positifnya untuk melawan sel kanker paru-paru (Sen et al., 2013) serta berperan dalam mengatur sistem imun tubuh (Nguyen et al., 2016).

PENUTUP

Penelitian hingga sejauh ini telah berhasil mengungkapkan manfaat bunga telang untuk menyokong kesehatan manusia melalui berbagai peran dan mekanisme, serta mengisolasi dan mengidentifikasi berbagai komponen bioaktif baik yang bersifat hidrofilik maupun lipofilik. Hasil-hasil penelitian ini memperkuat posisi bunga telang sebagai bahan untuk pangan fungsional dan nutrasetikal. Potensi bunga telang ini didukung pula oleh beberapa keunggulan bunga telang baik...
yang meliputi aspek budidaya, kestabilan ekstrak, maupun mutu nderawi.

Serangkaian penelitian, baik yang meliputi identifikasi peran masing-masing komponen bioaktif bunga telang terhadap efek fungsional tertentu, efek sinergistik yang ditimbulkan oleh kombinasi bunga telang dengan bahan lain, maupun uji klinis masih perlu dilakukan sebagai bagian dari persiapan pemanfaatan bunga telang sebagai bahan pangan fungsional dan nutrasetikal.

Merujuk kepada status terkini sesuai dengan penelitian yang telah dilakukan, pengolahan bunga telang sebagai minuman pengontrol gula darah adalah yang paling dekat dengan penerapan komersial.

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THE POTENCY OF CAJUPUTS CANDY IN MAINTAINING
THE COMPETITIVE CAPACITY OF STREPTOCOCCUS SANGUINIS
UPON STREPTOCOCCUS MUTANS

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ABSTRACT

Streptococcus mutans were competing Streptococcus sanguinis in biofilm formation. As pioneer colonizer, S. sanguinis were able to control S. mutans growth. This study was aimed to explore the ability of sucrose and non-sucrose cajuputs candies (SCC and NSCC) in maintaining the antagonistic relationship between the indigenous oral flora when they grew as dual-species biofilms (S. sanguinis and S. mutans). SCC and NSCC contained cajuput and peppermint oils as the flavor which the volatile compounds had been identified. Unflavored sucrose candy and unflavored non-sucrose candy were prepared similarly to the SCC and NSCC, respectively, except the flavor addition. SCC, NSCC, unflavored sucrose candy, unflavored non-sucrose candy, and the control were exposed in vitro to the biofilms. The biofilm was examined for biofilm inhibition capacity, DNA amount, and the expression level of spxB mRNA. The biofilm inhibition by SCC and NSCC were higher than the unflavored ones and were significantly different compared to the control. The SCC and NSCC managed to decrease the total DNA amount in the biofilm, but unflavored candies did not. The qPCR assays showed that the exposure of candies did not alter the proportion of S. sanguinis DNA to S. mutans DNA in the biofilms. Meanwhile, spxB mRNA expression indicated the ability of S. sanguinis to control S. mutans growth.

Keywords: Biofilm; cajuputs candy; Melaleuca cajuputi; spxB gene; Streptococcus mutans; Streptococcus sanguinis.

ABSTRAK


Kata kunci: Biofilm; Melaleuca cajuputi; permen cajuputs; spxB gene; Streptococcus mutans; Streptococcus sanguinis.
INTRODUCTION

Dental caries is one of the most common diseases in oral cavity (Somaraj et al., 2017; Eslami et al., 2016). Eco-systemic factors such as saliva, dietary habit, and microbical composition in biofilm contribute to its formation (Fejerskov, 2004; Becker et al., 2002). In addition, interaction among polymicrobial could lead to cases of dental caries (Becker et al., 2002; Kreth et al., 2005). *Streptococcus sanguinis* is a health-associated species (Percival et al., 2006; Kreth et al., 2005) and is considered as beneficial bacterium in regards to dental caries (Percival et al., 2006; Kreth et al., 2005; Magalhaes et al., 2016). On the contrary, *Streptococcus mutans* is the most commonly found species on dental caries (Oda et al., 2015) because it manages to grow in acidic environment and produces acidic compounds by fermenting carbohydrates (Percival et al., 2006; Becker et al., 2002; Kreth et al., 2005). Interestingly, *S. sanguinis* is able to antagonize *S. mutans* activity and protects the host from the negative effect of this opportunistic pathogen (Kreth et al., 2009).

*S. sanguinis* pioneers the development of oral biofilm, which have a role for the healthy dental plaque (Kreth et al., 2009). This bacterium is able to produce hydrogen peroxide (H₂O₂) that inhibits *S. mutans* growth under aerobic condition (Zheng et al., 2011a). *S. mutans* is not able to tolerate H₂O₂ (Zheng et al., 2011b). The H₂O₂ production in *S. sanguinis* was generated by pyruvate oxidase (SpxB). SpxB activity was controlled by SpxB gene (Zheng et al., 2011a). Expression of *spxB* mRNA represented the activity of *spxB* gene that corelated with the production of H₂O₂ by *S. sanguinis* (Magalhaes et al., 2016). Therefore, the physiological activity of *S. sanguinis* to inhibit *S. mutans* can be evaluated by the expression level of *spxB* mRNA (Zheng et al., 2011a).

Cajuputs candy is an Indonesian herbal-based candy that has been developed since 1997 as a functional food. Cajuputs candy was invented to increase the economic value of cajuput oil from *Melaleuca cajuputi* plant and to create a novel and distinctive Indonesian food product. Cajuputs candy has been patented (ID 0000385 S) (Wijaya et al., 2002) and developed into several variants including Sucrose Cajuputs Candy (SCC) (Wijaya et al., 2011) and Non-Sucrose Cajuputs Candy (NSCC) (Iftari et al., 2013). Both SCC and NSCC have been produced and marketed in certain area in Indonesia with good consumer acceptance.

Based on the previous studies, it is known that SCC and NSCC could suppress the growth of several pathogenic microbes in the oral system such as *S. mutans*, *S. sobrinus* (Wijaya et al., 2011) and *Candida albicans* (Wijaya et al., 2014). Iftari et al. (2013) reported that NSCC showed inhibition on biofilm formation and *gfp* expression of *S. mutans* serotype c. Cajuputs candy activity could be caused by a synergistic effect between cajuput oil and peppermint oil, which were utilized as the main flavoring components. Cajuput oil contains α-terpineol and terpien-4-ol which had been reported having antimicrobial activity against *Streptococcus spp.* and *C. albicans* (Jedlickova et al., 1994). Peppermint oil, which contains menthol as the major component, had been also reported for its antimicrobial activity against cariogenic bacteria (Dwivedi et al., 2012; Galvao et al., 2012).

Antibiofilm potency of cajuputs candy formulas toward single type of pathogenic bacteria that related to dental caries has been proven (Wijaya et al., 2011; Iftari et al., 2013). However, its effect on polymicrobial biofilms, especially *S. sanguinis* and *S. mutans*, has not been reported. The aim of this study was to determine the ability of SCC and NSCC to maintain the competitive capacity of *S. sanguinis* toward *S. mutans*. The effect of cajuput oil and peppermint oil as flavor and the effect of sucrose and non-sucrose as the raw material were validated in vitro by biofilm assay, quantification of *spxB* mRNA expression level and total bacterial DNA using qPCR technique.

MATERIALS AND METHODS

Materials

Food grade cajuput oil distilled from *M. cajuputi* was obtained from Pulau Buru Maluku and sweeteners such as sucrose, liquid glucose, isomalt and acesulfame-K were obtained from local supplier while peppermint oil and honeydew flavor were from flavor houses. Analytical grade cajuput oil was distilled from *Melaleuca cajuputi* plant and was utilized as the main flavoring component. Analysis grade sucrose and non-sucrose were used as the raw materials. Analytical grade peppermint oil was used as flavor and the effect of sucrose and non-sucrose as the raw material were validated in vitro by biofilm assay, quantification of *spxB* mRNA expression level and total bacterial DNA using qPCR technique.
chemicals were used: Brain Heart Infusion (BHI) agar and liquid (Acumedia), distilled water, milli-Q water, hexane (JT Baker), alkane standard (C₈-C₂₀), Nitrogen, carbon dioxide, violet crystal 1%, ethanol 95% (Merck), ethanol p.a. (Merck), phosphate buffer saline (PBS), trizol reagent (Invitrogen), isopropanol (Merck), chloroform, SYBR green (Kapa Biosystems), ultrapure distilled water (Invitrogen) and RT-PCR primer (1st BASE Custom Oligos, Singapore).

**Preparation of candy formulas**

Four candy formulas in this experiment are: (1) Unflavored sucrose candy, (2) Sucrose Cajuputs Candy (SCC), (3) Unflavored non-sucrose candy, and (4) Non-Sucrose Cajuputs Candy (NSCC). SCC and NSCC were prepared based on the procedures conducted by Wijaya et al. (2002) and Iftari et al. (2013), respectively. Similar procedures were conducted by removing flavor (cajuput and peppermint oil) to prepare unflavored sucrose and non-sucrose candy. For in vitro assay, all candy formulas were diluted 1:1(w/v) aseptically in sterile BHI broth. They will be used in biofilm inhibition assay and qPCR analysis. BHI broth without candy formula was used as a negative control.

**Volatile compounds identification**

SCC or NSCC (50 grams) were diluted with 20 mL distilled water and 5 mL hexane. They were put on a shaker (160 rpm) for 15 hours until all candies were dissolved. The supernatant was pipetted then added with sodium sulphate anhydrous. This water-free flavor extract was then pipetted into a new vial and added with 0.5 mL of 1,4-dichlorobenzene 1% (diluted with hexane) as an internal standard. The final extract solution was flushed with N₂.

Each extract (0.5 mL) was injected into the GC-MS (Agilent) on split mode (50:1 for SCC and 5:1 for NSCC) that was equipped with a DB-5 capillary column (60m length; 0.25mm i.d.; 0.25µm film thickness; helium carrier gas). The injector temperature was 250°C, and detector was 280°C. The initial oven temperature of the column was 60°C (held for 5 min), increased to 250°C at 10°C/min and held constantly for 2 min. Alkane standard C₈-C₂₀ (Fluka) was used as an external standard.

Qualitative identification of the constituents was performed by comparison of their linear retention indices (LRI) with the literature and their mass spectral data (NIST library) (Muchtaridi et al., 2010; Adams, 2009). The volatile profiles of SCC and NSCC were compared to volatile profile of cajuput and peppermint oil from Iftari et al. (2013).

**Bacterial strains, media, and culture conditions**

*S. sanguinis* ATCC 10556 and *S. mutans* XC from -70°C culture stocks were grown in BHI agar in an anaerobic jar under microaerophilic condition (CO₂ 10%, N₂ 80%, H₂ 10%) and incubated for 24 hours at 37°C. For in vitro assay, the bacteria were harvested and adjusted to achieve 0.477 optical density in 490 nm (OD₄₉₀) for *S.sanguinis* and 0.061 in OD₅₅₀ for *S. mutans*, which equaled to 1 × 10⁶ colony forming units (CFU) mL⁻¹.

**Preparation of dual-species biofilm**

Preparation of dual-species biofilm for in vitro assay was conducted based on the method of Kreth et al. (2008) with modification. Two types of microplates were used, 96-well microtitre plate for biofilm inhibition assay and 6-well microtitre plate (Takara, Tokyo, Japan) for qPCR analysis (mRNA and DNA quantification). Briefly, 100 µL of *S. sanguinis* suspension (1×10⁶ CFUmL⁻¹) was inoculated into 96-well microplate and 350 µL (1×10⁶ CFUmL⁻¹) of the same culture was inoculated into 6-well microplate. They were incubated for two hours in anaerobic jars under a microaerophilic condition (CO₂ 10%, N₂ 80%, H₂ 10%) at 37°C. In order to prepare dual-species biofilms, 100 µL (1× 10⁶ CFUmL⁻¹) and 350 µL (1× 10⁶ CFUmL⁻¹) of *S. mutans* suspensions were respectively added into the 96 and 6-well microplate containing *S. sanguinis* biofilm, and re-incubated for 18 hours in a similar condition.

In regards to the inhibition assay, dual-species biofilm on 96-well microplate was added with 200 µL of diluted candies in BHI broth (1:1 v/v). For qPCR analysis, 6-well microplate biofilm was added with 700 µL of diluted candies. Biofilm
added with only BHI broth was used as the negative control. The microplates were incubated for 18 hours, after which, the medium in 96 and 6-well plates were decanted and the remaining planktonic cells were removed by rinsing the wells with PBS pH 7.2) three times.

**Biofilm inhibition assay**

The inhibition effect of candy formulas toward dual-species biofilm was analyzed using method described by Yamanaka et al. (2004) After PBS rinsing, the plates were air dried and the adhered bacteria was stained with 200 μL of 0.5% crystal violet (CV, Sigma Aldrich) for 15 minutes at 37°C. After rinsing twice with 200 μL of PBS, bound dye was extracted from the stained cells using 200 μL of 95% ethanol. The OD_{490} of the extracted CV was measured with a microplate reader (Bio-Rad Laboratories, Hercules, CA). The absorbance value OD samples compared to OD control. Each experiment was performed in triplicate and repeated two times in separated occasions.

**Bacterial DNA quantification**

Microbial DNA was extracted using Trizol® reagent, following the instruction provided by the company. The DNA concentration was determined by spectrophotometer and standardized prior to qPCR analysis. The qPCR mixture for DNA quantification (10 μL) included 5 μL SYBR Green 1x Universal (KAPA Biosystem), 1 μL DNA (100 μg/mL), 3.2 μL DEPC water, and 0.3 μL of 5 mM forward and reverse real-time PCR primers that was Ss 16S rRNA, Sm 16S rRNA, and universal primers of 16S rRNA gene (Table 1). The qPCR protocol included one cycle of 95°C for 3 minutes, followed by 40 cycles of 95°C for 3 second and 60 minutes for 30 second. The bacterial load was determined based on the proportion of each species compared with total bacteria. It was determined by using the ∆Ct method (Yoshida et al., 2003).

**Analysis of the expression of spxB mRNA**

RNA extraction was performed in similar procedures with DNA extraction. It was reversed to cDNA using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Moreover, reactions mixture (10 μL) which contained 1xKAPA SYBR Green Master Mix (KAPA Biosystems), 1 μL cDNA, forward and reverse primers (0.5 μM) of spxBSs was centrifuged at 2000 rpm for 1 minutes. The housekeeping gene (16S rRNA) was used as internal control. All primers used in this study are shown in Table 1. The real-time PCR cycle was carried out under the similar conditions as previous DNA quantification. The relative change in spxB mRNA expression was analyzed using 2^(-ΔΔCt) (Suzuki et al., 2005).

**Statistical analysis**

Student’s t test (SPSS Inc 17.0 software) was carried out to analyze the data significance on a p-value of <0.05 among the biofilms OD, spxB mRNA expression, and bacterial proportion of experimental samples to the control groups. One-way analysis of variance (ANOVA) by SPSS Inc 17.0 was used in biofilm inhibition analysis with significance value p<0.05. ANOVA analysis was carried out to support the assumption/discussion about significance biofilm inhibition between samples.
THE POTENCY OF CAJUPUTS CANDY IN MAINTAINING THE COMPETITIVE CAPACITY OF STREPTOCOCCUS SANGUINIS UPON STREPTOCOCCUS MUTANS

Table 1. Primers used for qPCR

<table>
<thead>
<tr>
<th>Primer Sequence (5’-3’)</th>
<th>Purpose</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sm 16S rRNA forward CCACTCGGGAGGCAGCAGTAG</td>
<td>S. mutans quantification</td>
<td>(Shemesh et al., 2007)</td>
</tr>
<tr>
<td>Sm 16S rRNA reverse CAACAGAGCTTTACGATCGGAAA</td>
<td>S. mutans quantification</td>
<td>(Shemesh et al., 2007)</td>
</tr>
<tr>
<td>Ss 16S rRNA forward CCGCCCTAAGGTGGGATAGATGATTG</td>
<td>spxB mRNA and S. sanguinis quantification</td>
<td>(Zheng et al., 2011a)</td>
</tr>
<tr>
<td>Ss 16S rRNA reverse ACCTTCCGATA CGCGGACTTTAGGTG TACCGGCTACAAGG</td>
<td>spxB mRNA and S. sanguinis quantification</td>
<td>(Zheng et al., 2011a)</td>
</tr>
<tr>
<td>spxB Ss forward AATTCGCGCGGCTCAA</td>
<td>spxB mRNA quantification</td>
<td>(Zheng et al., 2011a)</td>
</tr>
<tr>
<td>spxB Ss reverse AAGGATAGCAAGGAA</td>
<td>spxB mRNA quantification</td>
<td>(Zheng et al., 2011a)</td>
</tr>
<tr>
<td>Universal forward TCTCACGGGAGGCA GCGT</td>
<td>Total bacteria quantification</td>
<td>(Suzuki et al., 2003)</td>
</tr>
<tr>
<td>Universal reverse GGACTACCAGGGGTAT</td>
<td>Total bacteria quantification</td>
<td>(Suzuki et al., 2003)</td>
</tr>
</tbody>
</table>

RESULTS

Volatile compounds contents

Volatile compounds of SCC and NSCC are presented in Table 2. The major compounds in SCC were 1,8-cineole and α-terpineol, while in NSCC were menthol and α-terpineol. Both SCC and NSCC contained 1,8-cineole, α-terpineol, β-caryophyllene, terpinen-4-ol, menthol, menthone, limonene, β-pinene, α-terpineine, γ-terpineine, and viridiflorol. Monoterpene groups, especially oxygenated monoterpenes, dominated these bioactive components.

Inhibition of dual species biofilm by Cajuputs candy

SCC, unflavored sucrose candy, NSCC, and unflavored non-sucrose candy were evaluated for their effect on the dual-species biofilm formation. Figure 1 shows that after incubation, both sucrose and non-sucrose candy showed inhibition activity.

The inhibition of all candy samples were significantly different from control (p<0.05). Moreover, the biofilm inhibition between samples were observed. OD values with different subset (a, b, ab, and c) mean they were significantly different with each other. As could be seen from Figure 1, the inhibition of SCC and NSCC were significantly higher compared with unflavored sucrose candy indicated the lower growth of the tested bacteria in biofilm mass of SCC and NSCC. The addition of cajuput and peppermint oils as flavoring ingredients in SCC and NSCC significantly reduced the biofilm formation by more than 50% compared to the control (growth medium cultured with bacteria without any formula addition). Meanwhile, the inhibition of SCC was not significantly different from unflavored non-sucrose candy (p>0.05). Biofilm inhibition was also observed in unflavored sucrose candy. Biofilm inhibition of unflavored sucrose candy was significantly lower compared to the control.
The potency of cajuputs candy in maintaining the competitive capacity of Streptococcus sanguinis upon Streptococcus mutans

Wijaya, C.H., Sari, B.R.E, Bachtiar, B.M.

Table 2. Chemical composition of volatile compounds of Sucrose Cajuputs Candy (SCC) and Non-Sucrose Cajuputs Candy (NSCC)

<table>
<thead>
<tr>
<th>No.</th>
<th>Compounds</th>
<th>LRI Refa</th>
<th>SCC</th>
<th>NSCC</th>
<th>References for antimicrobial activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α-pinene</td>
<td>939</td>
<td>940</td>
<td>0.98</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>β-pinene</td>
<td>979</td>
<td>984</td>
<td>0.86</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>p-cymene</td>
<td>1026</td>
<td>1031</td>
<td>1.63</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>D-limonene</td>
<td>1029</td>
<td>-</td>
<td>-</td>
<td>1039 1.92 (Maggi et al., 2009)</td>
</tr>
<tr>
<td>5</td>
<td>1,8-cineole</td>
<td>1031</td>
<td>1043</td>
<td>23.67</td>
<td>1044 8.48 (Maggi et al., 2009 ; Hamoud et al., 2012)</td>
</tr>
<tr>
<td>6</td>
<td>γ-terpinene</td>
<td>1059</td>
<td>1064</td>
<td>2.78</td>
<td>1065 0.51 (Inouye et al., 2001)</td>
</tr>
<tr>
<td>7</td>
<td>Terpinolene</td>
<td>1088</td>
<td>1094</td>
<td>1.9</td>
<td>1095 0.66 (Inouye et al., 2001)</td>
</tr>
<tr>
<td>8</td>
<td>Linalool</td>
<td>1096</td>
<td>1098</td>
<td>0.36</td>
<td>1100 0.34 (Dwivedi et al., 2012 ; Maggi, 2009)</td>
</tr>
<tr>
<td>9</td>
<td>Isopulegol</td>
<td>1145</td>
<td>1156</td>
<td>0.32</td>
<td>1158 0.63 (Inouye et al., 2001)</td>
</tr>
<tr>
<td>10</td>
<td>Menthone</td>
<td>1152</td>
<td>1165</td>
<td>3.3</td>
<td>1168 4.43 (Inouye et al., 2001)</td>
</tr>
<tr>
<td>11</td>
<td>Isomenthone</td>
<td>1162</td>
<td>1175</td>
<td>2.91</td>
<td>1178 4.34 (Inouye et al., 2001)</td>
</tr>
<tr>
<td>12</td>
<td>Menthol</td>
<td>1171</td>
<td>1185</td>
<td>7.29</td>
<td>1191 13.32 (Jedlickova et al., 1994 ; Inouye et al., 2001)</td>
</tr>
<tr>
<td>13</td>
<td>Terpinen-4-ol</td>
<td>1177</td>
<td>1189</td>
<td>1.86</td>
<td>1194 1.28 (Jedlickova et al., 1994 ; Maggi, 2009)</td>
</tr>
<tr>
<td>14</td>
<td>Neoisomenthol</td>
<td>1186</td>
<td>1195</td>
<td>0.26</td>
<td>1199 0.51 (Dwivedi et al., 2012 ; Inouye, 2001)</td>
</tr>
<tr>
<td>15</td>
<td>α-terpineol</td>
<td>1188</td>
<td>1203</td>
<td>9.17</td>
<td>1209 9.97 (Dwivedi et al., 2012 ; Inouye, 2001)</td>
</tr>
<tr>
<td>16</td>
<td>Pulegone</td>
<td>1237</td>
<td>1252</td>
<td>0.47</td>
<td>1256 0.92</td>
</tr>
<tr>
<td>17</td>
<td>Piperitone</td>
<td>1252</td>
<td>1267</td>
<td>0.29</td>
<td>1271 0.73</td>
</tr>
<tr>
<td>18</td>
<td>Menthy acetate</td>
<td>1295</td>
<td>1299</td>
<td>1.37</td>
<td>1302 2.56</td>
</tr>
<tr>
<td>19</td>
<td>α-terpinyl acetate</td>
<td>1354</td>
<td>1359</td>
<td>3.66</td>
<td>1362 3.38</td>
</tr>
<tr>
<td>20</td>
<td>α-copaene</td>
<td>1376</td>
<td>1398</td>
<td>0.34</td>
<td>1400 0.39</td>
</tr>
<tr>
<td>21</td>
<td>β-elemene</td>
<td>1390</td>
<td>1409</td>
<td>0.32</td>
<td>1412 53</td>
</tr>
<tr>
<td>22</td>
<td>β-caryophyllene</td>
<td>1419</td>
<td>1452</td>
<td>6.78</td>
<td>1456 7.64 (Heleno et al., 2011)</td>
</tr>
<tr>
<td>23</td>
<td>α-humulene</td>
<td>1454</td>
<td>1475</td>
<td>4.2</td>
<td>1489 4.67</td>
</tr>
<tr>
<td>24</td>
<td>Viridiflorol</td>
<td>1592</td>
<td>1631</td>
<td>0.57</td>
<td>1635 0.77 (Iscan et al., 2002)</td>
</tr>
</tbody>
</table>

Note:
a: LRI reference (Adams, 2009) with DB-5 column; b: LRI experiment with DB5-MS column.

Figure 1. Inhibition of candies exposures on dual-species (S. sanguinis and S. mutans) biofilms
Bacterial DNA quantification: effect of candy exposure to the relative amount and the proportion of *S. sanguinis* and *S. mutans* in dual-species biofilm

Table 3 showed the quantification of relative amount of total DNA in the four formulas compared to the total DNA in the control group (defined as 100% growth). Our data showed that the exposures of SCC and NSCC decreased the amount of total bacterial DNA significantly, indicating the involvement of the flavor. In contrast, when the unflavored candies were exposed into the bacterial biofilm, the amount of bacterial DNA were significantly increased compared to those of control. Nevertheless, the DNA proportion of *S. sanguinis* and *S. mutans* in the biofilm did not show any significant difference between samples and control (Table 3). This study showed that the exposure of the candies did not alter the antagonistic interaction between *S. sanguinis* and *S. mutans*, *in vitro*.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Relative amount of total DNA bacteria (%)</th>
<th>Proportion</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>S. sanguinis</em> (%)</td>
<td><em>S. mutans</em> (%)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100.00</td>
<td>41.47</td>
<td>58.53</td>
<td></td>
</tr>
<tr>
<td>Unflavored sucrose candy</td>
<td>184.77</td>
<td>35.58</td>
<td>64.42</td>
<td></td>
</tr>
<tr>
<td>SCC</td>
<td>1.20</td>
<td>35.39</td>
<td>64.61</td>
<td></td>
</tr>
<tr>
<td>Unflavored non-sucrose candy</td>
<td>221.29</td>
<td>36.35</td>
<td>63.65</td>
<td></td>
</tr>
<tr>
<td>NSCC</td>
<td>1.04</td>
<td>38.95</td>
<td>61.05</td>
<td></td>
</tr>
</tbody>
</table>

SCC: Sucrose Cajuputs Candy, NSCC: Non-Sucrose Cajuputs Candy

No significance difference in proportion was observed between sample and control in the analysis of student’s t test with *p>*0.05

Effect of candy exposure on the expression level of *spxB* mRNA in the biofilms

Figure 2 showed that the exposure to all sample formulas did not eliminate the expression of *spxB* gene. No statistical difference was found between the expression of samples and the control group, which meant that the ability of *S. sanguinis* in producing H$_2$O$_2$ as its competing agent could be maintained. The *spxB* expression of NSCC and unflavored non-sucrose candy were higher than SCC.
DISCUSSION

Volatile profile

The volatile profiles of SCC and NSCC showed a similarity but vary in percentage (Table 2). Both candies were using cajuput and peppermint oil as the flavor. The flavor concentration in these candies were about 0.9%, that consisted of cajuput oil (0.7-0.8%) and peppermint oil (0.1-0.2%) based on their original formulations. Different heating temperature during production of SCC and NSCC may caused variation in their volatile percentage (Iftari et al., 2013). Table 2 showed that oxygenated monoterpenes (1,8-cineole, α-terpineol, menthol, menthone, and terpinen-4-ol) were dominated the bioactive volatiles in SCC and NSCC. Functional groups of bioactive compounds might contribute to their antimicrobial activities. As reported by Inouye et al. (2001), the antimicrobial activity of terpene alcohols (menthol, α-terpineol, and terpinen-4-ol) were higher than terpene ketone (menthone) as well as terpene ether (1,8-cineole), and terpene hydrocarbons (limonene, β-pinene, and γ-terpinene) were lowest in activity.

Biofilm inhibition

This study showed cajuputs candy exposures suppressed the formation of dual-species (_Streptococcus sanguinis_ + _S. mutans_) biofilms. In comparison to the control, cajuputs candy (SCC and NSCC) were more effective in inhibiting biofilm formation than the unflavored candies (Figure 1). Meanwhile, biofilm inhibition of unflavored sucrose candy was significantly lower compared to the control, despite of the missing flavor as anti-bacterial agent. The potency of cajuput and peppermint oils as antibiofilm agent was due to their bioactive volatiles. Cajuput and peppermint oils contain highly hydrophobic substances, such as 1,8-cineole, menthol, and menthone (Table 2). These volatile components have been reported for their antimicrobial capacity (Inouye et al., 2001; Maggi et al., 2009; Hamoud et al., 2012; Iscan et al., 2002; Jedlickova et al., 1994; and Dwivedi et al., 2012). Direct contact of these oils to the biofilm at long period of incubation (over 18 hours) could inhibit biofilm growth. The inhibition mechanism can be vary by disturbing membrane function, inhibition of cells respiration, and alteration of ion transport processes which lead to the death of microbial cell (Hamoud et al., 2012), thus can reduce biofilm formation. The biofilm inhibition of NSCC was higher compared to SCC, which meant that the combination of flavor and non-sucrose carbohydrate in NSCC may play a role. However, further study is needed to elucidate the exact mechanism of SCC and NSCC in inhibiting biofilm formation.

*p<0.05* indicated statistically significant difference between sample and the control group.
Not only NSCC and SCC, the unflavoured non-sucrose candy in comparison with the control also exhibited a significant inhibition in biofilm formation. As shown in Figure 1, the inhibition of unflavored non-sucrose candy was not significantly different from SCC. Lower biofilm density produced when biofilms were exposed to non-sucrose candies than that were exposed to sucrose candies. It showed that the usage of isomalt to substitute sucrose in the candy formulation exhibited biofilm inhibition. In the present study, different kind of carbohydrate were used, and this may promote different biomass density. Therefore, the usage of isomalt in non-sucrose candy formulations will produce lower biomass density in comparison with sucrose or glucose containing candies (Mayo and Ritchie, 2009). Sucrose is naturally more available to be fermented by microorganism than isomalt (Childers et al., 2011).

The unflavored candies were also reported to have lower biofilm density than control (Figure 1). In this study, the unflavored sucrose candy contained high amount of sucrose and glucose, while the unflavored non-sucrose candy contained high amount of isomalt. The sucrose and isomalt concentration were approximately 50% of the candy formulations (data not shown), meanwhile the growth medium (BHI broth) itself already contained sucrose and glucose. The high amount of such carbohydrates (e.g. sucrose and isomalt formulations) in the biofilm may cause environmental disturbance to bacterial growth. High content of external carbohydrates exposed to the biofilm will modify the growth environment, increases osmotic pressure, that will disturb bacterial growth (Touger-decker and Loveren, 2003). These disturbances were able to reduce the ability of bacteria to form biofilm.

In this study, the result of biofilm inhibition was presented by optical density (OD) value. OD value sometimes represents number of bacterial cell, but in term of biofilm, it does not merely indicating bacterial cell, but biofilm mass. Biofilm is a complex mixture of several materials consisting of bacterial cells, proteins, carbohydrates, water, lipids, including non-viable cell of bacteria that form a biofilm mass (Kreth et al., 2008). It means that OD value also represented biofilm density. Biofilm can have low OD value, eventhough its bacterial number (DNA) was high. As it can be shown, a contradictionary result was found between OD value (Figure 1) and DNA amount (Table 3) of unflavored candies compared to the control.

**DNA quantification**

Both unflavored sucrose candy and unflavored non-sucrose candy were able to decrease biofilm formation compared with control (Figure 1). Meanwhile, higher bacterial DNA was detected in those unflavored candies (Table 3). It seemed that OD values from the biofilm inhibition measurement could not be used to distinguish the amount of bacterial load. Since the crystal violet assay did not only determine the number of bacterial cells but also measure the extracellular matrix within biofilm, low OD value did not mean low bacterial number. OD value is the determination of turbidity, which represents the biomass density of the biofilm (Bakke et al., 2001). Therefore, in contrast with crystal violet assay, qPCR is a sensitive method to quantify total and/or individual bacterial DNA in clumping cells, including the presence of viable and non-viable cells (Childers et al., 2011). Moreover, it can also be used to quantify specific bacterial species (Fortin et al., 2001).

In this study, unflavored non-sucrose candy had the higher number of bacterial DNA and even higher than that in unflavored sucrose candy (Table 3). This result indicated that isomalt was used in biofilm metabolism. However, isomalt can still be used in bacterial metabolism, thus can support bacterial growth. Mayo and Ritchie (2009) reported that the incubation time of 18-24 hours implied isomalt degradation by *Streptococci* isolates used. Frequent and prolonged contact of isomalt (an equimolar mixture of D-glucopyranosyl-1,6-sorbitol and α-D-glucopyranosyl-1,6-mannitol) used in “sugar-free” cough drop syrup could be degraded to produce glucose, sorbitol, and mannitol.

This study showed that NSCC had the greatest antibacterial activity and inhibition toward biofilm formation (Figure 1), which was significantly different from the control. NSCC inhibited biofilm formation by approximately 68.2% compared to...
control and decreased bacterial DNA to 1% of the control (Table 3). These phenomena were due to antibacterial volatiles provided from the flavor, supported by the limitations of the substrate. Essential oils tend to inhibit more powerfully on Gram-positive bacteria than the growth of Gram-negative bacteria, which could be attributed to the different structure and cell membrane compositions (Guiterrez et al., 2008). S. mutans and S. sanguinis, which are Gram-positive bacteria, have single membrane structure with thick peptidoglycan layer that are sensitive to essential oil (Trombetta et al., 2005).

The DNA proportion was measured to determine the effect of cajuputs candy formulas (SCC and NSCC) on the ability of S. sanguinis to maintain its antagonistic competency upon S. mutans growth. Both SCC and NSCC were able to decreased bacterial load in the biofilm (Table 3) and inhibited biofilm growth (Figure 1), but the result of DNA proportion (Table 3) indicated that they did not show any capacity to interfere the natural interaction among S. mutans and S. sanguinis. The absence of cajuputs candy increased the number of both bacterial in biofilm. On the contrary, their presence simultaneously inhibited (Table 3). Kreth et al. (2005) reported that S. sanguinis and S. mutans have a competitive exclusion in the biofilm system caused by the production of diffusible substances that inhibited the other growth. S. sanguinis produces peroxidase, while S. mutans produces bacteriocin. This study showed that, the cajuputs candy exposures did not interfere such competitiveness effect (Table 3). Thus, the exposures may have a capability in maintaining the antagonistic competency of S. sanguinis upon S. mutans in the biofilm.

**Gene Expression of spxB mRNA**

We have demonstrated that the presence of cajuputs candy in biofilm development resulted in up-regulating of spxB mRNA expression, which implied the transcription activity of spxB gene of viable S. sanguinis. The spxB gene produces an enzyme responsible for production of H₂O₂ by S. sanguinis to inhibit the growth of S. mutans (Zheng et al., 2011a). The detectable expression of spxB mRNA in all of the tested formula (Figure 2) indicated the competitive capacity of S. sanguinis against S. mutans and showed that viable bacteria remained within the biofilms. No negative effect was found in the expression of spxB mRNA compared to control since there were no statistically difference between samples and control (Figure 2). This indicated that the bacterium still has capacity in controlling S. mutans as naturally occurred in vivo (Kreth et al., 2008).

The exposure of unflavored sucrose candy showed the lowest expression level of spxB mRNA compared to others formulas (Figure 2), whereas the amount of total DNA bacteria was very high (Table 3). Kreth et al. (2005) mentioned that in high sucrose concentration, S. sanguinis preferred to use the carbon source for growth rather than spend a lot of energy to produce H₂O₂ to suppress the growth of the S. mutans.

The spxB mRNA expression of that in NSCC exposure was higher than SCC exposure (Figure 2). The use of isomalt on NSCC might create a stress condition due to the lack of carbon source that could be metabolized (Lemos et al., 2005). Therefore, under these conditions, S. sanguinis would focused on producing H₂O₂ to maintain the bacteria coexistence (Kreth et al., 2005), and increase the bacterium competitive capacity in the biofilm (Zhu and Kreth, 2012).

It is known that the essential oil is a mixture of various components that are mostly hydrophobic compounds and have antibacterial properties. The mechanism essential oil biofilm inhibition were very diverse but mostly related to disruption of membrane function by penetration of hydrophobic compounds (Nazaro et al., 2013). Other proposed mechanisms include the alteration of the membrane fatty acids, degradation of cell wall, alteration of the proton motive force, increase of permeability of the membrane, and membrane protein damage (Nazaro et al., 2013). Even though SCC and NSCC exposure effectively inhibited the dual-species biofilm formation (Figure 1), the inhibition did not eliminate the ability of S. sanguinis to produce H₂O₂, as proven by the up-regulation of spxB mRNA expression because S. sanguinis is less susceptible to bioactive compounds in essential oil than S. mutans (Magalhaes et al., 2016). Therefore,
THE POTENCY OF CAJUPUTS CANDY IN MAINTAINING
THE COMPETITIVE CAPACITY OF STREPTOCOCCUS
SANGUINIS UPON STREPTOCOCCUS MUTANS

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the survival S. sanguinis still has the ability to express the spxB gene. However, more studies are needed to fully understand the inhibition mechanism of cajuputs candy in the dual-species biofilm formation.

CONCLUSIONS

The data showed that the absence of flavor in the candy triggered greater biofilm growth than the control. Flavored candy exposures effectively inhibited the bacterial growth within the biofilms. Each tested candy showed the ability to maintain the antagonistic competency of S. sanguinis toward S. mutans in dual-species biofilms. The combination of active volatiles and non-sucrose material made NSCC exposures as the most effective formula to inhibit the biofilm growth without eliminating its competitive capacity of S. sanguinis toward S. mutans in dual-species biofilm. However, more studies are needed to fully understand the inhibition mechanism of cajuputs candy in polymicrobial biofilm formation. The data in this study can be used for further exploration of the SCC and NSCC potency in preventing dental caries in vivo.

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ABSTRACT

Indonesia has been accounted as one of the biggest palm oil producers in the world with annual production capacity reaching over 34 million tons. As a consequence, the amount of wastes resulting from this industry requires immense attention to be given. One of the wastes resulted is the Palm Fatty Acid Distillate (PFAD), which in previous researches has been proven to contain some beneficial bioactive compounds such as squalene. Squalene is known as one of the best natural emollients for pharmaceuticals and cosmetics, so that many researches have given the attempt to extract squalene from PFAD. Despite all attempts, large amount of impurities such as free fatty acids (FFA) were still to be found present in squalene extract. Therefore, in this research an effort to enhance the pre-treatment process of PFAD was done by combining saponification process and centrifugation, in order to remove FFA prior to extraction process. Three different pre-treatment scenarios in single stage liquid-liquid extraction (LLE) were compared in their effect on squalene content found in the extract using GC-MS analysis. The analysis showed that the squalene content increased from 5.370 to 9.320 % (w/w) when centrifugation was applied. Adding another round of saponification to this method has increased the content even further to 23.940 %. Furthermore, the application of multiple stage extraction could increase the squalene content to 37.450 %.

Keywords: Liquid-liquid extraction; multiple stage extraction; Palm Fatty Acid Distillate; squalene.

ABSTRAK

Indonesia merupakan salah satu negara penghasil minyak sawit terbesar di dunia, dengan kapasitas produksi melebihi 34 juta ton per tahun. Dengan besarnya volume produksi minyak sawit, jumlah limbah yang dihasilkan pun sangat besar dan memerlukan perhatian khusus. Salah satu limbah industri minyak sawit adalah Distilat Asam Lemak Minyak Sawit (PFAD), yang telah dibuktikan mengandung beberapa senyawa bioaktif, di antaranya adalah squalene. Squalene merupakan salah satu krim alami terbaik untuk pengobatan dan kosmetik, sehingga banyak upaya telah dilakukan untuk mengekstrak squalene dari PFAD melalui proses bertingkat. Akan tetapi, beberapa pengotor seperti asam lemak bebas masih ditemukan dalam ekstrak squalene yang diperoleh. Oleh karena itu, dalam penelitian ini dilakukan upaya untuk mengoptimalkan proses perlakuan awal, dengan menggunakan teknik separasi untuk mengurangi jumlah asam lemak sebelum ekstraksi dilakukan. Dari percobaan ini, ekstraksi tingkat tertinggi memperlihatkan hasil analisa GC-MS kandungan squalene yang meningkat dari 5,370 ke 9,320 % (b/b) jika dilakukan sentrifugasi. Penambahan proses penyabunan pada metode ini juga meningkatkan kandungan skualen lebih lanjut ke 23,940 %. Dengan penggunaan ekstraksi bertingkat jamak dapat meningkatkan kandungan skualen menjadi 37,450 %.

Kata kunci: Distilat Asam Lemak Minyak Sawit (PFAD); ekstraksi bertingkat jamak; ekstraksi cair- Cair; squalene.
INTRODUCTION

In recent years, palm oil has become one of the most potential resources of vegetable oils in the world. Indonesia has been one of the biggest countries that produces palm oil which yields over 30 million tons yearly where 80 % is exported. There are several waste products, which come from the production of palm oil such as empty fruit bunches, palm press fiber, palm oil mill effluent, palm kernel cake, palm kernel shell, and sludge cake (Prasertsan and Prasertsan, 1996). Due to the high production rate of palm oil in Indonesia, the high amount of palm oil waste offers high potency to be utilized. One of the wastes that is generated through the refinery process of crude palm oil is Palm Fatty Acid Distillate (PFAD), which can be used as feedstock for animal feed, soap industry, oleo chemical industry and combustion for local power generation (Zero & Rainforest Foundation Norway, 2015).

PFAD was also found to contain some beneficial bioactive compounds, among those is squalene (Yusuf, et. al., 2015), which can be used mainly for food supplements and pharmaceutical ingredients. In addition, it also can be considered as one of the best natural emollients in pharmaceuticals and cosmetics (Gapor, 2010). Moreover, squalene can be applied as a detoxification factor, as an eye and skin antioxidant. It has been widely known, that the demand for squalene is increasing from time to time, while there is limited availability from conventional sources such as shark liver oil. At the time being, the availability of shark liver oil is low due to many regulations prohibiting the killing of sharks. With this condition, the interest to get squalene from other sources has been arisen. Obtaining squalene from PFAD became very favorable to be explored, despite several obstacles faced related to its purity (Gapor, 2010).

Based on a previous study (Yusuf, et. al., 2015), squalene extract can be obtained by using organic solvents such as dichloromethane (DCM) through liquid-liquid extraction (LLE). Wandira, et. al. (2017) has proposed an optimum condition for the saponification and extraction process to extract squalene from PFAD, resulting in an extract with a squalene content of around 24 %. In a separate study, Sibuyo, et. al. (2017) has applied multiple stage LLE to extract squalene from PFAD using DCM, and has proven that compared to a single stage extraction, this method could increase squalene content up to 1.33 times, depending on the ratio used between PFAD and DCM. However, in these researches, the content of FFA in the extract was still considerably high. It could be assumed, that the saponification process conducted prior to LLE, was not able to remove the majority of FFA contained in the PFAD. Hence, this research was aimed at enhancing the pre-treatment process, and to study the effect of incorporating different sequences of PFAD pre-treatment methods on the squalene content in the extract.

MATERIALS AND METHOD

Materials

The materials used in this research are including PFAD sample, L – ascorbic acid (Merck, Germany), potassium hydroxide (Merck, Germany), ethanol 96 % (JT-Baker), dichloromethane (Mallincrodt, USA), iso-propanol (Mallincrodt, USA), toluene (Mallincrodt, USA), and distilled water. The sample of PFAD was taken from a palm oil refinery industry in Bekasi, Indonesia.

Research Methodology

In this experiment, several pre-treatment methods were applied (Figure 1). It comprised of three different research paths, which were then followed with acid base titration analysis. Research path 1, is principally the suggested squalene extraction method from previous researches (Sibuyo, et. al., 2017 & Wandira, et. al., 2017), whose results this research aimed to improve. In research path 1, PFAD would go under a saponification process prior to a liquid-liquid extraction, with the purpose to as much as possible reduce the amount of FFA, by converting it into glycerol and soap. After saponification, LLE process would proceed using dichloromethane (DCM) as a solvent, where afterwards, the extracted sample was analyzed by using gas chromatograph – mass spectroscopy (GC-MS) analysis for the determination of squalene content.
In the second and third research paths, a combination between saponification and centrifugation was applied, prior to LLE. Centrifugation is a process that can separate a mixture based on density differences by applying centrifugal force field. The product of saponification between PFAD and KOH are mainly glycerol and soap, with the rest of unreacted FFA and impurities contained in the mixture. Given that the density of glycerol was 1.26 g/cm$^3$ and the density of soap is 0.932 g/cm$^3$, centrifugation was considered to be a practical method to separate the two phases. Free fatty acid, with a density of 0.961 g/cm$^3$ is expected to be found in a bigger portion in the bottom layer together with glycerol, while squalene with a density of 0.858 g/cm$^3$ should be found in the upper layer together with the soap. This way, it is expected that adding a centrifugation process after saponification will give favor to the subsequent process, which is the extraction of squalene.

Multiple stage extraction process with 3 stages was applied in the latest part of the experiment in order to determine how much increase of squalene content can be achieved by using a combination of the selected pre-treatment scenario and the multiple stage extraction process.
Pre-Treatment Process

The pre-treatment process will comprise saponification and centrifugation processes. As much as 10 g of PFAD sample was added to 0.500 g of ascorbic acid in the three necks round bottom flask. Then, 88 ml of 96 % ethanol was added into the mixture. Heating was applied to maintain the temperature at 70˚C by using water bath while being continuously stirred using magnetic stirrer. The mixture was saponified with 10 ml of 50 % w/v concentration of potassium hydroxide (KOH) using reflux condenser in water bath for 60 minutes. Then, centrifugation of the mixture occurred twice at 3,000 rpm and 5,000 rpm for 10 and 30 minutes. In the research path 3, the saponification procedure was repeated after the centrifugation process.

Extraction of Sample

Single Stage Extraction

The sample and 100 ml of distilled water was poured into 500 ml separator funnel at ± 25˚C and shook in vertical direction carefully. Following this step, 75 ml of dichloromethane was added into the mixture and left for approximately an hour until two separated layers were formed. The transparent layer of the mixture was removed and the remaining liquid was mixed with another 75 ml of dichloromethane and left for an hour for extraction. The transparent layer was removed once again to be collected with the previous obtained extract. This repetition was conducted three times, and the total volume of the transparent layer was measured and labelled as Extract 1.

Multiple Stage Extraction

The multiple stage extraction was conducted following the schematic diagram shown in Figure 2. Each circle in this figure represents a single extraction process, with the exact procedure to be performed as explained previously. The numbers written in the circles show different separator funnels used in each step. The procedure for multiple stage extraction as shown above is a means to batch-wisely approximate a continuous multiple stage process. The first three stages shown in the diagram is the pre-conditioning stage, where stages 4 until 6 are considered to be the approximated real condition. Hence, the analysis of squalene content was done on extracts E9, E12 and E15, and additionally also on extract E1, so that a comparison between squalene content prior and subsequent to multiple stage extraction can be made.
Analysis Techniques

Titration Acid-Base Analysis

In order to determine the free fatty acid percentage in oil, titration acid-base analysis based on ASTM D 974 (American & Standard 2003) was conducted. This is the standard test method for acid and base number by color indicator titration. This method can be used to indicate the acidic or base constituents in petroleum products and lubricants that are soluble in mixtures of toluene and isopropanol. Titration was conducted in this study by the addition of 50 ml of iso-propanol and 50 ml of toluene into the sample in 250 ml conical flask. The addition of 20 drops of napthol benzene indicator into the solution then followed, and this mixture was titrated with standard alkali solution (potassium hydroxide 0.087M), while being vigorously rotated until dark green color was observed. The volume of standard KOH was used to determine the free fatty acid value.

Gas Chromatograph – Mass Spectroscopy (GC-MS) Analysis

Gas Chromatograph – Mass Spectroscopy (GC-MS) analysis was used to analyse the squalene content of the resulting extracts. The column that was used was HP Ultra 2 Capillary Column Length x Internal Diameter x Film Thickness = 30 m x 0.25 mm x 0.25 µm. Helium (He) gas was used as the carrier gas. The initial temperature of oven was set at 70°C and held for 0 minute, then rising at 3°C/min to 150°C. The instrument was then injected with 1 µl sample with the constant flow of 0.9 µl/min. Thus, it was being on hold for 1 minute and finally rising at 20°C/min to 280°C and was held for 26 minutes. The temperature was set for injection port at 250°C, ion source at 230°C, interface at 280°C and quadruple at 140°C. The detector used was coupled to mass spectrometry.

RESULTS AND DISCUSSION

Optimization of pre-treatment process

The extraction method by Sibuyo, et. al. (2017) & Wandira, et. al. (2017), which only suggested a single saponification method as a pre-treatment prior to LLE, was to be improved through addition of centrifugation and another round of saponification in this research. In order to analyze whether there is a decrease in FFA content in the PFAD sample in each path, titration acid-base analysis was conducted. The volume of KOH added to change the titrated sample color into dark green was used to calculate the FFA content (%-w).

![Figure 3. Free fatty acid content (%-weight) from different pre-treatment scenario](image-url)
The result summarized in Figure 3 shows that by applying centrifugation after saponification process (research path 2), the FFA content can be reduced to less than half of its initial amount. The FFA content has decreased from $3.36 \pm 0.03\%$ to $0.945 \pm 0.135\%$ when a centrifugation at 5,000 rpm for 30 minutes took place subsequent to saponification process. Adding another round of saponification subsequent to the centrifugation process (research path 3) has shown further improvement in removing FFA. Research path 3, which applied a combination between centrifugation and double saponification, has shown results with lowest FFA content ($0.3 \pm 0.06\%$) and therefore is proven to be able to remove a large portion of FFA contained in PFAD.

In order to confirm that the application of research path 3 is not only going to remove FFA but will as well have an effect on the squalene content in the final extract, the observation was continued by performing single stage extraction subsequent to the pre-treatment. Squalene content analysis of the resulting extracts was then conducted by means of GC-MS analysis.

All extract samples resulting from the single LLE process and the squalene standard were injected into GC-MS to obtain the chromatograms and mass spectrums. The result of GC-MS was observed qualitatively by chromatogram, including the quantitative analysis by the area under each peak which was shown in the chromatogram. Based on the chromatogram of squalene standard as shown in Figure 4 below, the retention time of squalene was shown at 26.107 min. Therefore, there should be a peak with similar retention time in the chromatogram if the extract does contain squalene. An example of GC-MS analysis result on one extract is shown in Figure 5, where a similar peak to the squalene standard could be observed at an approximately same retention time. This indicates qualitatively that this extract indeed contains squalene. Afterwards, a quantification of the amount of squalene present in the extract was done by determining the area below the peak.

![Figure 4. Chromatogram of squalene standard at retention time 26.107 min](image-url)
Figure 5. Chromatogram of an extract (research path 2 at 5,000 rpm and 30 minutes) at retention time 26.085 min indicating the existence of squalene.

The three different pre-treatment scenarios performed previously were observed further to study their effects on the squalene content in the extract through GC-MS analysis and the results of these analyses are depicted in Figure 6.

Figure 6. Squalene content in extracts resulting from 5 experiments based on GC-MS Result. Experiment 1: saponification process with 50 %-w/v KOH continued by LLE process (representing the research path 1). Experiment 2 and 3: saponification process with 50 %-w/v KOH, continued with centrifugation at 5,000 rpm for 30 minutes, and LLE (representing research path 2 in two replications). Experiment 4 and 5: saponification process with 50 %-w/v KOH, followed by centrifugation at 5,000 rpm for 30 minutes, second step of saponification with 50 %-w/v KOH, and LLE Process (representing research path 3 in two replications).
Figure 6 shows that the squalene content in the extract resulting from single stage extraction was increased from 5.370% (research path 1) to 9.320% (research path 2). However, the highest squalene content was found in experiment 4 (research path 3), where up to 23.940% squalene content was obtained. Even though the replication of GC-MS analysis of the same research path (experiment 5) did not deliver as high squalene content as in experiment 4, it still showed an increase in squalene content compared to other experiments.

There are several reasons why research path 3 delivered highest squalene content. This research path combined a centrifugation and double saponification as pre-treatment of PFAD, and was proven to be able to remove a large portion of FFA in it. After the implementation of this combination of pre-treatment, the FFA content was reduced down to 0.3 ± 0.06%. During extraction, squalene was expected to be more soluble in DCM, while soap phase will be distributed more in water phase. The extraction solvent DCM was chosen, because it had been proven by Yusuf (2015) to be the best solvent in extracting squalene from PFAD. However, FFA is also found to be highly soluble in non-polar organic solvent (Astuti, et al., 2010), so that it might also be found in DCM phase during extraction. Hence, reducing the amount of FFA as much as possible prior to the extraction will favor the selectivity of squalene.

Moreover, the centrifugation was also capable in removing a large portion of glycerol and soaps, which are not desired to enter the LLE process. Removing these impurities has been proven to have significant effect on the squalene content in the final extract. Hence, based on this result, an optimum pre-treatment process for squalene extraction from PFAD is decided to follow the research path 3, which combines a centrifugation and double saponification process.

**Multiple Stage Extraction**

Multiple stage process (Figure 2) was applied in this study in order to determine how far the squalene content can be increased by applying the previously chosen pre-treatment method. The first three stages were the preliminary stages, and for the analysis, stage 4 until 6 were observed. Each circle of the process represents a single stage extraction conducted in a separator funnel, where layers of extract and raffinate would be formed. The extract resulted from stage 4 to 6 were then analyzed using GC-MS to determine their squalene content. Additionally, extract coming from the first step was also analyzed in order to make a before-and-after comparison of the squalene content. The GC-MS results of these extracts are summarized in the Figure 7 below.

![Figure 7. Comparison of squalene content in single and multiple stage extraction based on GC-MS result](image-url)
As mentioned in previous section of this paper, extract E1 was analyzed to represent the single LLE using research path 3 as pre-treatment, whereas extracts E9, E12 and E15 were analyzed to represent the results of multiple stage LLE. The extract E15 is considered as the resulting final extract approximating real condition in a continuous multiple stage LLE process. Even though the experiment labelled with Single Stage Path 3 in Figure 7 was conducted with the exactly same procedure with E1, the GC-MS result showed slightly different value in squalene content. This could be caused since there was possibly a slight difference in the PFAD sample, as the waste specification taken from the palm oil industry might differ from day to day, depending on many factors during the production process.

The final squalene content resulting from the multiple stage process (extract E15) was 37.450 %, which was significantly higher than the squalene content in the extract obtained from single-stage extraction, which was 26.600 %. It has been shown, that the application of pre-treatment processes combined with multiple stage extraction was proven to be able to significantly increase the squalene content in the extract. However, the removal of FFA prior to the LLE process did not necessarily reduce its content in the extract to the lowest possible amount. A relatively large amount of FFA was still detected in the GC-MS analysis results, which were mostly the oleic and palmitic acid. In order to reduce the FFA content in the extract even further, hence, increasing the squalene content, the application of other separation techniques need to be explored. An extract purification must also be taken into consideration, in order to remove impurities from the squalene extract.

CONCLUSION

This research has proven that by applying centrifugation process in a combination with double saponification, the free fatty acid (FFA) content in PFAD can be reduced down to 0.3 ± 0.06 %-w. Further combination between this pre-treatment and a multiple stage extraction process was found to be able to increase the squalene content in the extract up to 37.450 %-%. Compared to a single stage extraction with saponification alone as pre-treatment, this number shows an increase of squalene content of around six folds. It was also concluded that despite this positive result, there exists a big room for improvement to be explored in future works. The impurities were still found as majority in the extract, and mostly was identified as free fatty acids. This indicates that the removal of FFA in the pre-treatment must be supported also with extract purification at the end.

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IMPROVING METHODOLOGY OF SQUALENE EXTRACTION FROM PALM FATTY ACID DISTILLATE (PFAD) THROUGH ENHANCED PRE-TREATMENT PROCESS

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EXTRACTION OF HYALURONIC ACID FROM ALOE BARBADENSIS (ALOE VERA)

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ABSTRACT

Hyaluronic acid have a high moisture preservation and biocompatibility characteristic, thus allowing various usage of this substance in pharmaceutical, medicinal, and skin care products. Present manufacturing process of hyaluronic acid requires extraction of animal cells or through other methods utilizing bacteria. The aim of this research is to investigate an alternative source of hyaluronic acid extraction using plant based which is Aloe barbadensis (aloe vera). Three main parts of aloe vera (rind, mesophyll and gel) underwent several steps of extraction process and the result was compared to the sample of hyaluronic acid from goat brain. The evaluation including comparison of total carbohydrates, reducing sugars and degradation using heat treatment. The results of extraction were analyzed using UV-Spectrophotometer at 230 nm and compare to the extraction result of goat brain to ensure the presence of hyaluronic acid. It was found out that the rind part of aloe vera have the highest potential of high content of hyaluronic acid.

Keywords: Aloe vera; extraction; hyaluronic acid.

ABSTRAK

Asam hialuronat memiliki kemampuan untuk mempertahankan kelembapan serta biokompatibilitas yang tinggi, hal ini menjadi alasan asam hialuronat banyak digunakan dalam produk farmasi baik yang berhubungan dengan obat maupun perawatan kulit. Pada proses pembuatan asam hialuronat, ekstraksi dari sel hewan masih merupakan sumber utama disamping penggunaan metode alternatif menggunakan beberapa jenis bakteria. Tujuan dari penelitian ini adalah untuk menginvestigasi sumber alternatif penggantian asam hialuronat menggunakan bahan dasar tumbuhan yaitu Aloe barbadensis (lidah buaya). Terdapat tiga bagian dari lidah buaya yang melewatinya beberapa tahap ekstraksi (kulit, mesofil, dan jel), hasil dari ekstraksi kemudian dibandingkan dengan sampel asam hialuronat dari otak kambing. Evaluasi mencakup perbandingan karbohidrat total, penurunan kadar gula, dan degradasi molekul menggunakan panas. Analisis terakhir menggunakan UV-Spektrofotometer di panjang gelombang 230 nm dan dibandingkan dengan hasil ekstraksi dari otak kambing untuk memastikan keberadaan asam hialuronat. Hasil analisis menunjukkan bahwa kulit lidah buaya memiliki potensi mengandung asam hialuronat yang cukup tinggi.

Kata kunci: Asam hialuronat; ekstraksi; lidah buaya.
INTRODUCTION

Hyaluronic acid (HA) is a biological occurring polymer which has substantial biological functions in almost every organism (Necas, et.al., 2008). In humans, HA can be found in skin, vitreous of the eye, umbilical cord, and synovial fluid, but it is also present in body’s tissues such as skeletal tissues, heart valves, lungs, brain, and many others (Meyer K., Palmer, J.W., 1934). Hyaluronic acid was located predominantly within extracellular and peri cellular matrix, although correspondingly existed on the intracellular cell (Balazs, et al., 1986).

Resources to gain hyaluronic acid were commonly taken from various animal tissues such as human umbilical cords, rooster combs, bovine vitreous humor, and bovine synovial fluid (Liu, et. Al., 2011). At present day, even though production through animal-based tissues still remain unshaken to be the major pathway for large HA production, another possibility of production systems have been demanded because of some disadvantages of the existing process. Due to the grinding procedure and several repetition of using acid and organic solvents, both practical and mechanical issues will always happened in animal extraction in terms of cost and safety ( Widner, et. al., 2005).

Another issue is that HA from animal tissues may remain connected to a HA-specific binding cellular proteins of hyaluronidase (Fraser, et al., 1997). Hyaluronidase is undesirable since it may trigger the risk to prohibit an immune response. Furthermore, transmitter of infectious diseases in form of nucleic acids, prions, and viruses may well increases within extraction procedure ( Shiedlin, et al., 2004). Lastly, the procedure are expensive and require a long period of time, labor, and advanced facilities to accommodate processes involved from animal extraction until purification of HA (Shlini, et al., 2017). Hence, it is preferred to generate hyaluronic acid via an animal cell-free system that could reduce contagion of undesirable contaminant and expense of manufacturing ( Widner, et al., 2005) and (Yu & Stephanopoulos, 2008). Therefore, this research was arranged to find another pathway of extracting hyaluronic acid from a plant source, which according to (Shlini, et al., 2017) has proved to be successfully done from sweet potato and tapioca (Sana, et al., 2017). Moreover, aloe vera (A. barbadensis) was chosen due to its popularity to the public and considerably easy to be harvested in Indonesia.

In this research A. barbadensis is chosen as the potential source of HA due to similarities with HA in compositions and biological activities. Both aloe vera and hyaluronic acid proven to promotes wound healing (including dermatology applications), anti-inflammatory and therapeutic benefits. Moreover, A. barbadensis and hyaluronic acid have been used for dermatology purposes due to their abilities to retain water. There are three major parts of A. barbadensis used in this research, those are: rind, mesophyll, and gel. Rind is the external surface waxy cuticle which performs as a wall in a contradiction to moisture loss. Rind covers several levels of structures, with slight beneath from the waxy cuticle remains an area where the aloe related bacteria live (Sushruta, et al., 2013). Mesophyll is a liquid yellow-brownish part of aloe vera which holds the xylem and phloem vascular bundles. Mesophyll has the biggest concentration of anthraquinones and chromones of the whole aloe vera. Last part of aloe vera is the gel which located inside the inner parenchyma part of aloe vera. It consist of two components: juice of the gel and fibrous pulp enriched with cellulose.

Commercial manufacturing of hyaluronic acid is built on either animal-based extraction or genetically modified strains of bacterial fermentation. Both of these pathways are commonly applied and proved to manufacture hyaluronic acid products with molecular weights above 10 kDa that was suitable for medicine and dermatology usage (Liu, et al., 2011). Biological properties of hyaluronic acid are connected with its molecular weight, hence there is a great interest in HA degradation and evaluation of the biological behavior of HA fragments. Mechanisms of the HA cleavage into its smaller fragments involve enzymatic, free radical, thermal, ultrasonic, and chemical methods such as acid and alkaline hydrolysis (Soltes, et al., 2007).
MATERIALS AND METHOD

Materials

All aloe vera (A. barbadensis) and fresh goat brain were purchased from a market in Tangerang, Indonesia. The chemicals used for this research were acetone (Amresco), chloroform (Merck), methanol (FULLTIME), sodium acetate (CV. Bina Sejahtera), L-cysteine (Merck), acetic acid (Merck), 37% hydrochloric acid (Sigma Aldrich), ethylenediaminetetraacetic acid/EDTA (Disolvins), distilled water, sodium chloride (HiMedia Laboratories), absolute ethanol (FULLTIME), sulfuric acid (J.T Baker), ice cubes, sea salt, sodium carbonate (Merck), anhydrous sodium acetate (Merck), sodium hydroxide (Merck), potassium sodium tartrate tetrahydrate (PUDAK Scientific), dinitrosalicylic acid/DNS (Sigma Aldrich).

Equipment

M254A BEL Engineering Weighing balance, water filtration system (Hydro Water Solution PT. Hydro Water Technology), hotplate stirrer (WiseStir MSH-20D), MColorpHast pH-indicator strips, centrifuge (Type 80-2 China), refrigerator (Electrolux), autoclave HG 50 Hirayama, Phillips food processor/grinder, PG Instruments T60 UV-Visible Spectrophotometer, and VWR V-1200 Visible Spectrophotometer.

Extraction Process

The extraction methodology is based on the studies being performed by (Shilini, et al., 2017) with sweet potato (Ipomoea batatas) and (Sana, et al., 2017) with tapioca (Manihot esculenta). In this research, aloe vera (A. barbadensis) will be taken as the plant source and goat brain as sample of pure hyaluronic acid. The samples were washed thoroughly, parts of aloe vera were separated by knife and each of the four samples were homogenously crushed. 50 g of each sample was submerged in 50 mL of acetone and stirred for an hour. Chloroform and methanol with ratio 2:1 was used to incubate 100 mL sample for 24 hours at 25°C. Followed by digestion buffer (100mM sodium acetate pH 5.0, 5.0mM cysteine and 5.0mM disodium EDTA) that arranged in a ratio 2 mL of buffer to 100mg of tissue. The sample was hydrated inside the digestion buffer for 44 hours at 5°C before centrifuge at 3200rpm for 30 minutes. The solvents was removed and the solid filtrate was splashed by 3 mL of 2.0M sodium chloride and followed by absolute ethanol. Absolute ethanol was inserted in ratio of 2:1 and kept for 24 hours at -16°C. The next procedure was centrifugation at 3200 rpm for 30 minutes. Sequentially, the supernatant was taken away and the solid filtrate was washed with 80% ethanol. Second centrifugation was done as previous one before supernatant was discarded and the solid filtrate dried for 24 hours at 25°C. The final solid was re-suspended in 5 mL of distilled water and stored inside a test tube.

Total Carbohydrate Analysis using Anthrone’s Method (Hodge, et al., 1962)

0.1 g of sample was boiled for 3 hours with 5 mL of 2.5N-HCl, then cooled to room temperature with ice and salt. The sample was neutralized by adding solid sodium carbonate until the effervescence ends. The sample was made up to the volume of 100 mL and centrifuged at 3200 rpm for 15 minutes. The supernatant was collected to prepare 1mL aliquots for analysis. The sample was added by 4mL of fresh anthrone reagent (dissolve 0.2 g of anthrone in 100 mL of ice cold H2SO4) and heated for 8 minutes in a boiling water. The sample was rapidly cooled with ice and sea salt and observed at absorbance of 630 nm in a visible spectrophotometer.

Reducing Sugar Analysis using DNS Method (Garriga, et al., 2017)

DNS reagent was prepared by making two mixtures; Solution A (1 g of DNS was dissolved in 20 mL of NaOH 2M) and Solution B (30 g of potassium sodium tartrate tetrahydrate was dissolved in 50 mL of distilled water). Solution A was added into Solution B, heated, and mixed on a hot plate at 300°C and 370 rpm. This new solution was completed to the volume of 100 mL with distilled water and stored in amber bottle at refrigerator (4°C). This solution was named as DNS reagent. 1 mL of each sample was placed into a test tube and added by 1 mL of DNS reagent. The test tube was heated in a boiling water for 5 minutes and cooled.
by ice and sea salt to room temperature. The sample was added by 8 mL of distilled water and read at 540 nm in a visible spectrophotometer.

**Fragmentation of Hyaluronic Acid (Lowry and Beavers, 1994) and (Botner, et al., 1988)**

Degradation of the pre-assumed HA sample and goat brain sample were done through thermal degradation. 10 mL of each sample was taken into small bottle and inserted into the autoclave for 4 hours at 128°C. Sequentially, the sample was observed using UV-spectrophotometer in 230 nm wavelength.

- **RESULTS AND DISCUSSION**

  **Total Carbohydrate Measurement**

  Hyaluronic acid is a carbohydrate compound, more specifically a repeated glycosaminoglycan (GAG) which formed of β4-glucuronic acid and β3-N-acetylglucosamine (Meyer K, 1934). Hyaluronic acid occurred in a high molecular weight due to the repetition of glucuronic acid and N-acetylglucosamine that able to goes up to a thousand repetition even further as can be seen from figure 1.

  ![Figure 1. Structure of hyaluronic acid monomer (Cowman & Matsuoka, 2005)](image)

  Anthrone’s method was used to measure total carbohydrate content from three different part of aloe vera samples (rind, mesophyll and gel) to be compared to total carbohydrate content of hyaluronic acid from natural source, in this case goat brain. This method used as the initial stage to identify hyaluronic acid.

  As can be seen from Figure 2, all of aloe vera’s parts (rind, mesophyll, and gel) were proved to show some value of absorbance at 630 nm, which showed that aloe vera does contains carbohydrate.

  Goat brain as the hyaluronic acid source showed highest peak with the value of absorbance of 0.034 followed by rind with absorbance of 0.023. From three parts of aloe vera (rind, mesophyll and gel), rind part showed highest and closest absorbance to hyaluronic source from goat brain, but to be certain further analysis through reducing sugar needs to be done.
Reducing Sugar Measurement.

Anthrone method only cover the general picture of finding carbohydrate, hence another method is used to observe more specific compositions of carbohydrate which downgrade the structure from polysaccharides into smaller fragments of carbohydrates; reducing sugar. Moreover, hyaluronic acid chemical structure is particularly included a form of reducing sugar: β-D-glucose (Gunawardena, 2015), which made the essential on doing DNS is highly proposed.

Figure 3. β-D-glucose (Gunawardena, 2015)

DNS method was done as a complement procedure from anthrone’s result to specifically qualify any reducing sugars inside the sample. Total carbohydrate analysis through anthrone’s methods already showed that rind and goat brain has highest and closest absorbance compared to other part of aloe vera. Figure 4 below showed that all parts of A. barbadensis have shown value of absorbance which suggested contains reducing sugar. It should be highlighted that both in anthrone and DNS method, rind part of aloe vera showed the highest absorbance 0.333 in comparison to mesophyll and gel. In addition, rind have the closest absorbance to goat brain (0.288) that contain high concentration of hyaluronic acid in both anthrone and DNS method thus conforming that rind have a very high chance to contain hyaluronic acid. Based on these findings, rind was chosen to undergo further analysis step which is thermal degradation.
Hyaluronic acid is naturally occurred in a high molecular weight, but since there are various applications which came from different sizes of molecular weight of hyaluronan, it prompted a HA cleavage method to be performed. There were numerous ways to decrease the molecular weight of hyaluronic acid into smaller fragments which engage with enzyme, free radical, heat, ultrasound, and chemicals. Unfortunately, most of those methods will produce unwanted toxic impurities and demand a high cost. Thermal degradation of hyaluronic acid proved to be successfully done by (Botner, et al., 1988) at 128°C in an autoclave. Based on total carbohydrate and reducing sugar measurement, rind has the highest chance of containing hyaluronic acid, hence thermal degradation analysis was done to conforming the presence of hyaluronic acid in rind compared to goat brain. Hyaluronic acid was proved to be existed on the wavelength of 230 nm based on several studies being done by (Shlini, et al., 2017) and (Sana, et al., 2017). Therefore, the rind sample and goat brain were gone through UV-spectrophotometer before and after thermal degradation to showed the existence of hyaluronic acid.
Hyaluronic acid is naturally occurred in a high molecular weight, but since there are various applications which came from different sizes of molecular weight of hyaluronan, it prompted a HA cleavage method to be performed. As can be seen from Figure 5 and Figure 6, three repetitions of both samples showed a decrease of absorbance with very similar value, hence showed degradation process using heat treatment to be successful and hyaluronic acid component from both samples was successfully fragmented as the end product. One law that affirm molecular weight of the end product after degradation will decreased is the law of conservation of mass. The law stated that mass is neither created nor destroyed in chemical reactions (Sterner, R and Hood, J., 2011). Since thermal degradation was not a chemical reaction, it only shrinks the structure molecules which produced a less bulky compound with smaller weight of mass.

Another supportive evidence to show the declining of its molecular weight is the smaller value of the concentration after degradation procedure. If the chemical structure of HA were cut during thermal degradation, it ends with less bulky chemical compounds which leads to smaller value of concentration. The concentration of the sample was declined after degradation as can be seen in the decreased of absorbance value. This can be explained through the Lambert Beer’s Law, expressed through:

\[ A = \varepsilon c l \]  
(Equation. 1)

Whereas A is absorbance, \( \varepsilon \) is molar absorption coefficient, c is molar concentration and l is optical path length passed by the UV light. Since the value of absorbance after thermal degradation was lower compared from before degradation process, it concluded that concentration after degradation was also dropped due to proportionally equivalent value of absorbance and concentration according to the Equation. 1. It can be clearly seen that the drop of concentrations was constant through three repetitions of sampling using UV-spectrophotometer which referring back to Figure. 5 and Figure. 6.

This result also supported by the fact that rind is highly composed by one of the hyaluronic acid structures; carboxyl group which are richly present in form of oxalic acid inside rind. Moreover, rind has anti-inflammatory property due to chromones which someway equaled with hyaluronic acid’s anti-inflammation property. Chromones also have skin protection effects which matched with one of hyaluronic acid’s benefits for skin; protection of water loss to the skin. Lastly, on just below the waxy cuticle of rind, there is an area where aloe correlated bacteria live. Gram-positive microbes (including Group A and group C Streptococci) which able to produce hyaluronic acid through bacterial pathway, were only found on the surface of aloe vera (A. barbadensis), whereas coccobacilli (streptococcus morbillorum, enterococcus faecium, and other Gram-negative rods) are observed only in gel part.

CONCLUSION

Anthrone method showed that all parts of aloe vera containing carbohydrate with rind has the highest absorbance, just below the absorbance of goat brain. This result was confirmed by DNS method which showed that again rind has the highest absorbance just like the goat brain. Furthermore, thermal degradation process was done to degrade high molecular weight HA into small molecular weight HA. The result of thermal degradation can be seen through UV-Spectrophotometer which showed constant and very similar decrease of absorbance on both rind and goat brain sample, thus showed that rind is containing hyaluronic acid. For further studies, isolation and purification of hyaluronic acid and quantification of its concentration, ion exchange chromatography is preferred due to anionic nature of hyaluronic acid. The elution obtained by ion exchange chromatography can be further purified using gel permeation chromatography and for determination of precise structure of HA, NMR (Nuclear Magnetic Resonance) followed by FT-IR can be used in future research.
REFERENCES


CHOCOLATE BAR WITH MORINGA AND DATES AS CALCIUM-RICH FOOD WITH LOW GLYCEMIC INDEX FOR ENDURANCE ATHLETES

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ABSTRACT

This research aims to provide chocolate for endurance athletes by utilizing the potential of highly nutritious local foods. The ingredients used are chocolate, moringa leaves (Moringa oleifera), and dates (Phoenix dactylifera). This research method uses a Completely Randomized Design (CRD) with One Way Anova statistical analysis. Samples were analyzed by testing in a food laboratory. In this study, moringa powder was used for its calcium content and dates were used for its carbohydrates content with a low glycemic index. In 100 grams of developed chocolate contains 3.27 g of water, 1.83 g of ash, 30.4 g of fat, 5.32 g of protein, 59.1 g of carbohydrate, 0.38 g of crude fiber, 427.07 mg of calcium.

Keywords: Chocolate; dates; endurance athletes; low GI; moringa.

ABSTRAK

Penelitian ini bertujuan untuk menyediakan cokelat bagi atlet endurance dengan memanfaatkan potensi pangan lokal yang bernilai gizi tinggi. Bahan-bahan yang digunakan adalah cokelat, daun kelor (Moringa oleifera), dan kurma (Phoenix dactylifera). Metode penelitian ini menggunakan Rancangan Acak Lengkap (RAL) dengan analisis statistik One Way Anova. Sampel diuji di laboratorium pangan. Dalam penelitian ini, penggunaan moringa powder didasari oleh kandungan kalsiumnya dan kurma oleh kandungan karbohidratnya yang memiliki indeks glikemik rendah dalam 100 g cokelat yang dikembangkan adalah sebagai berikut: 3.27 g air, 1.83 g abu, 30.4 g lemak, 5.32 g protein, 59.1 g karbohidrat, 0.38 g serat kasar, 427.07 mg kalsium.

Kata kunci: Atlet endurance; cokelat; GI rendah; kelor; kurma.
INTRODUCTION

In the competition period, the supply of food must meet the quantity and quality of nutrition, namely the amount of energy and balanced nutritional composition. It is recommended that the consumption of carbohydrate-source foods as a reserve of muscle and liver glycogen needed during the match. This aims to prevent the occurrence of hypoglycemia, prevent fatigue and maintain muscle working power. Feeding needs to be arranged so that before the competition begins the process of food digestion is complete. This is important because, during the competition, blood flow is concentrated into the muscles to deliver nutrients and oxygen needed when the muscles contract. Usually, 2-3 hours before the match has given snacks such as bread/crackers (Welis and Syafrizal, 2009). Refill carbohydrate deposits with a pre-event meal or snack for 1-4 hours before the competition around 1-4 g / kg BB (Louise Burke and Greg Cox, 2010).

In this case, the concept of the glycemic index (GI) was developed, which is a level of food according to its effect on blood sugar levels. Consumption of foods with low GI (± 2 hours before a competition) can guarantee the release of glucose into the bloodstream constantly during the game. This is because foods with a low GI are digested slowly so that their storage is also slow. Extra glucose will be available until the end of the game because muscle glycogen is stored slowly (Welis and Syafrizal, 2009). Low GI has characteristics that can cause the digestive process in the stomach to run slowly, so the rate of emptying the stomach (gastric emptying rate) also takes place slowly. This results in a suspension of food that has undergone digestion in the stomach (chyme) more slowly reaching the small intestine so that further digestion of carbohydrates and absorption of glucose in the small intestine occurs slowly. Likewise, in low-GI foods, most glucose uptake occurs in the upper small intestine (duodenum) and the middle part (jejunum). In the end, fluctuations in blood glucose levels are relatively small. With these metabolic characteristics, low-GI foods can reduce the glycemic and insulin responses (Hoerudin, 2012). Dates contain high carbohydrates with a low glycemic index so that it is potentially a food source of energy for endurance athletes. Dates are useful as a substitute for the energy lost when competing because of the natural sugar content. So, it does not make blood sugar pressure soaring sharply. Dates also contain potassium which can strengthen muscle function so that it is not easily injured. 100 g of dates can provide 314 kcal of energy with the main components being monosaccharides (Dayang et al., 2014).

High-intensity exercise causes a decrease in bone mass. Calcium plays an important role in bone health, especially for athletes who prioritize physical contact (Nguyen, 2010). Adequate calcium consumption helps maintain healthy bones and reduces the risk of injury to bones when competing. Athletes are encouraged to consume calcium-fortified foods as an alternative source of calcium to meet calcium needs per day. Calcium helps optimize bone density and prevents osteoporosis in athletes (Amiruddin and Yusni, 2015). One source of calcium is found in Moringa leaves. Moringa leaves are referred to as magic plants based on their use, especially related to medicine and nutrition. In 100 g fresh Moringa leaves contain at least 1077 mg of calcium (Fahey, 2005). The calcium content is increased when the Moringa leaves are dried.

Fortification can also be done on processed cocoa bean products, namely chocolate. Some types of chocolate products such as dark chocolate made from cocoa paste with the addition of a little sugar, milk chocolate made from cocoa paste, cocoa butter, sugar and milk powder, and white chocolate made from cocoa butter, sugar and milk powder. Also, there are couverture chocolates which are premium or high-quality chocolates that are often used by professionals in the industry to make pastries or cakes (Agus, 2012).

Nowadays athletes tend to want food they like and are practical, also consider food in terms of nutrition. The availability of local food-based snacks from chocolate, Moringa, and dates for athletes is still rarely found, especially in Indonesia. Therefore, it needs to be developed as an effort to utilize local food potential.
METHODOLOGY

Materials and Method

This study used an experimental method with a completely randomized design. Data analysis used One Way ANOVA and continued with the Duncan test. Samples were analyzed in a food laboratory, carbohydrate using the By Difference method, protein using the Kjeldahl method, fat using the Sokhlet method, crude fiber using Gravimetric method, ash using Gravimetric method, moisture using Gravimetric method, and Calcium using AAS method.

The ingredients consist of white chocolate, moringa powder and dates with a comparison of formulations in Table 1. The equipment consists of heat-resistant glass bowls, scales, chocolate molds, pans, spoons, aluminum foil, and refrigerators.

Steps to make chocolate with the addition of moringa flour and dates is chopped chocolate or cut it into small pieces to make it easy to melt, then put it in a heat-resistant glass bowl. Then melt the chocolate using the double boiler technique. By preparing a pan whose top surface fits with a chocolate bowl, then fill about half of the water, cook until it boils. Then place the chocolate bowl on the pan. This aims to keep the water vapor from mixing with chocolate because it will make the chocolate clot quickly and the texture is not good. Stir chocolate until melted. Turn off the heat, stir continuously then add the Moringa flour and dates that have been cut into small pieces. Then pour the chocolate into a mold and put it in the refrigerator until hard. The last, pack with aluminum foil.

Table 1. Comparison Formulations of Chocolate, Moringa, and Dates (g)

<table>
<thead>
<tr>
<th>Raw Material</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chocolate</td>
<td>40.00</td>
<td>40.00</td>
<td>40.00</td>
</tr>
<tr>
<td>Moringa Powder</td>
<td>5.00</td>
<td>10.00</td>
<td>15.00</td>
</tr>
<tr>
<td>Dates</td>
<td>15.00</td>
<td>10.00</td>
<td>5.00</td>
</tr>
</tbody>
</table>

Results and discussion

The results of ANOVA mean analysis presented in a pie chart. The pie chart explains the total average of nutritional content of chocolate bars in 100 grams.

Figure 1. Nutrition-related claims in chocolate bar products (Moisture)
Based on the analysis of nutrients found the highest moisture content is F2 3.67 g. The moisture content contained in the product is affected by the constituent ingredients. According to Rahmadi (2010), dates are a fairly high contributor to water content. A study by Sinaga et al., (2019) that the addition of Moringa leaf powder can increase ash content.

![Figure 2. Nutrition-related claims in chocolate bar products (Ash)](image)

Based on the analysis of nutrients found the highest ash content is F3 2.91 g. Because the content of Moringa powder is more than other ingredients. Moringa contains calcium that are high enough so that the more moringa powder, ash content will increase. About 96% of food consists of organic matter and water. The rest consists of mineral elements known as organic substances or ash content. Ash content indicates the mineral content contained in an ingredient (Fajri et al., 2013).

![Figure 3. Nutrition-related claims in chocolate bar products (Fat)](image)

Based on the analysis of nutrients found the highest fat content is F3 31.5 g. The fat content of this product is affected by the addition of dates and chocolate. White chocolate contains a high fat compared to other constituent ingredients. Fat in the body acts as an energy source, especially in sports with moderate intensity in a long time, for example endurance sports (Rismayanthi, 2015). According to Burke et al., (2004) fat is an important source of energy for muscle contraction during endurance sports. High-fat consumption (> 30% of total calories) can reduce carbohydrate intake, so muscle glycogen cannot be maintained. According to Fink and Mikesky (2015), the recommended consumption of fat for athletes per day is 20–35% of total energy, which includes 7–10% SFA, 10% MUFA, 10% PUFA.
Based on the analysis of nutrients found the highest protein content is F3 7.08 g. Because the concentration of Moringa powder is higher than other formulations. Athlete's protein requirements are in the range of 1.2–1.6 g/ kg body weight per day. Increased protein requirements because athletes are more at risk of damage to muscle tissue, especially when training or strenuous exercise (Irawan, 2007).

Based on the analysis of nutrients found the highest carbohydrate content is F1 59.1 g. Because of the concentration of dates more than other ingredients. Dates contain high carbohydrates with a low glycemic index so that it is potentially a food source of energy for endurance athletes (Hoerudin, 2012). Research by Hafidha (2018) that the addition of dates can increase carbohydrate levels. Research by Al-Shahib and Marshall (2003) that dates contain a high percentage of carbohydrates (total sugar, 44-88%). According to Louise Burke and Greg Cox (2010), the carbohydrate requirement of endurance athletes is 1–4 g per kg of body weight. Carbohydrates play a role in maintaining blood glucose levels and the speed of carbohydrate metabolism in the body to reduce the occurrence of fatigue in athletes who have endurance, carbohydrates contained in chocolate will be absorbed by the body slowly so that it can become a continuous source of glucose. Adding dates to this chocolate product can provide energy quickly for endurance athletes (Burke et al., 2004).
Based on the analysis of nutrients found the highest crude fibers content is F3 0.48 g. Because the concentration of Moringa powder is higher than other formulations. Besides, the addition of dates also contributed to the levels of the fiber of chocolate dates and moringa.

According to Rock (2009) dates contain 2.49–12.31% food fiber. The comparison between the addition of dates and moringa leaf powder affects the levels of crude fibers of chocolate moringa and dates.

Based on the analysis of nutrients found the highest calcium content is F3 783.6 mg. Because the concentration of Moringa powder is higher than other formulations. According to Mahmood et al., (2011) that Moringa contains good nutrients one of which is calcium. In 100 g of Moringa leaves contain at least 2003 mg of calcium (Fahey, 2005). Athletes who exercise with high intensity require calcium intake to maintain bone health to reduce the risk of injury to the bones during exercise or competition. According to Nguyen (2010) in the NSCA’s Performance Training Journal that the athlete’s calcium adequacy rate is Upper Level (UL) around 2500 mg/day.

CONCLUSION

Based on this research, F1 is the best formula. In 100 grams of developed chocolate contains 406.16 kcal, 3.27 g of water, 1.83 g of ash, 30.4 g of fat, 5.32 g of protein, 59.1 g of carbohydrate, 0.38 g of crude fiber, 427.07 mg of calcium. The final product is served as much as 30 grams. So, it contains ± 120 kcal, ± 18 g carbohydrates, ± 9 g fat, ± 1.6 g protein, ± 0.1 g crude fiber, ± 130 mg calcium. Based on the daily value of 2475 calorie diets, 30 grams of this product can meet the daily needs of calcium 10.6%, carbohydrates 5.2%, protein 2.2%, fat 10.9%. It can be concluded that this product can be a snack for endurance athletes with good nutritional content. Health claims may also contribute to the improvement of industrial competitiveness (Fadlillah et al., 2019). Chocolate with calcium content and low glycemic index is a new finding in the food industry, so it needs to be developed.
ACKNOWLEDGEMENT

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Reviewer Guideline

Interested to become a reviewer?

The JFFN Editorial Team will send the manuscripts to the relevant reviewers according to the expertise of respective reviewers. If you are interested in becoming a reviewer of JFFN, please fill out the reviewer application form: http://bit.ly/revregform along with a brief summary of your expertise and your CV. Send all the documents to jffn@sgu.ac.id. The reviewers who pass the selection will obtain many benefits. All review process will be processed through JFFN online system.

1. Confirmation (Accept or Decline)

   The reviewers will receive an email invitation that will be sent by the JFFN system. Use the links to accept or reject the invitation. If you decide to accept the invitation as the reviewer, you will be responsible to input the review result/s to the JFFN journal editor as the requirement whether the manuscript is appropriate to be published in JFFN.

2. Submitting the review

   Reviews must be entered in the JFFN submission system. Drop us an email if you encounter trouble accessing the manuscript or entering your comments to jffn@sgu.ac.id.

3. Timing

   The deadline for completing the manuscript review process is 14 days. If you are unable to complete or need additional time for the review process, please notify us immediately so that we can keep the authors informed and assign alternative solution if necessary.

Confidentiality

All reviewers are required to maintain the confidentiality of the manuscript and never share information to the other parties without the editor’s consent. The involvement of third parties in the review process, must be declared during the review process. Correspondence as part of the review process is also to be treated confidentially by all parties.

All reviewers are strongly required to keep the confidentiality of process reviews, maintain material confidentiality of manuscripts, and will not take advantage during the review process.

Anonymity

Reviewers are anonymous by default. Reviewers’ identities are not revealed to authors or to other reviewers unless reviewers specifically request to be identified by signing their names at the end of their comments.

All reviewer’s identity will be kept confidential. The reviewer's identity will not be revealed to anyone unless reviewers specifically request to notify the identity by writing the name on the review form or comments.

Writing the Review

Here are the generic questions to the reviewer. Please evaluate the submission based on the general scientific journal guideline. Please download the review form from: http://bit.ly/revformjffn.
The form covers:

1. Is the manuscript technically sound and do the data support the conclusion?
2. Has the statistical analysis been performed appropriately and rigorously?
3. Is the manuscript presented in an intelligible fashion and written in standard English/Indonesian?
4. Review comments to the author? Please state the positive suggestion that might support the authors to improve the manuscript.
5. If you would like your identity to be revealed to the authors, please include your name here (optional) *Your name will not be published in the manuscript.

Revisions

When an author revises a manuscript, the Academic Editor will often ask the original reviewer(s) to evaluate the authors’ revision. We expect the reviewers to be available to provide these additional comments. You will be requested to suggest the acceptance of the manuscript.

In the revision process, the editorial team frequently will ask reviewers to evaluate the author's revision. The editorial team expects that all reviewers will be available to conduct evaluation and provide valuable suggestions to improve the manuscript quality. In the end, the reviewer will be asked to decide the appropriateness of the manuscript according to several categories:

- Accept without revision
- Accept with minor revision
- Accept with major revision
- Decline
Thank you to our reviewers

Peer-review is an important step to maintain the high quality of a journal. Reviewers provide scientific critiques based on their expertise that assist editors to make acceptance decision professionally. Therefore, the Editors would like to acknowledge our reviewers listed below who have contributed their valuable time for maintaining the quality of Journal of Functional Food and Nutraceutical.

List of reviewers JFFN volume 01 no 02 February 2020:

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<thead>
<tr>
<th>Name</th>
<th>Institution</th>
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<tbody>
<tr>
<td>Adolf J. N. Parhusip</td>
<td>Universitas Pelita Harapan</td>
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<tr>
<td>Anastasia Fitria Devi</td>
<td>Pusat Penelitian Kimia Lembaga Ilmu Pengetahuan Indonesia (P2Kimia LIPI)</td>
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<tr>
<td>Andreas Romulo</td>
<td>Bina Nusantara University</td>
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<td>Azis Boing Sitanggang</td>
<td>IPB University</td>
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<tr>
<td>Della Rahmawati</td>
<td>Swiss German University</td>
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<td>Elisabeth Prabawati</td>
<td>Swiss German University</td>
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<tr>
<td>Erliana Ginting</td>
<td>Balai Penelitian Tanaman Aneka Kacang dan Umbi (Balitkabi), Kementerian Pertanian</td>
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<tr>
<td>Hendry Noer Fadlillah</td>
<td>International University Liaison Indonesia</td>
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<tr>
<td>Hery Sutanto</td>
<td>Swiss German University</td>
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<td>I Kadek Putra Yudha Prawira</td>
<td>IPB University</td>
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<td>Lisa Yakhin</td>
<td>Universitas Pelita Harapan</td>
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<td>Melanie Cornelia</td>
<td>Universitas Pelita Harapan</td>
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<td>Mutiara Pratiwi</td>
<td>Swiss German University</td>
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<td>Nina Artanti</td>
<td>Pusat Penelitian Kimia Lembaga Ilmu Pengetahuan Indonesia (P2Kimia LIPI)</td>
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<td>Nurul Asiah</td>
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<td>Phebe Hendra</td>
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<td>Silvya Yusri</td>
<td>Swiss German University</td>
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<td>Tabligh Permana</td>
<td>Swiss German University</td>
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Registrasi anggota P3FNI


Keterlibatan P3FNI atau ISFFN ini memberi manfaat anggotanya untuk memberikan kontribusi ilmu pengetahuan dan teknologi serta mengikuti pemutakhiran pangan fungsional dan nutrasetikal. Bersosialisasi dan berkesempatan dalam pertemuan ilmiah bersama penggiat pangan fungsional dan nutrasetikal dari berbagai negara untuk terus-menerus mengarah pada optimalisasi penggunaan pangan untuk kesejahteraan manusia secara bijaksana dan menjaga kelestariannya.

Siapa Yang Perlu Menjadi Anggota?

1. Akademisi dan peneliti yang terus-menerus mengembangkan dan memajukan ide alternatif dan kreatif untuk menuju kemajuan dan kesejahteraan manusia dengan menggunakan data basis ilmiah.
2. Praktisi kesehatan maupun industri yang menerapkan pangan fungsional dan nutrasetikal.
3. Mahasiswa sebagai penerus masa depan untuk melestarikan praktik-praktik pengadaan dan penggunaan pangan fungsional dan nutrasetikal secara bijaksana dan berkelanjutan.
4. Memberi advokasi dalam kasus-kasus pangan dan kesehatan.
5. Individu yang memiliki perhatian dalam pangan fungsional dan nutrasetikal.

Fasilitas Anggota P3FNI

1. Link Internasional untuk kegiatan atau program riset dan pembelajaran pangan fungsional dan nutrasetikal meliputi direktori laboratorium dan professor, lembaga kesehatan, LSM/NGO, skim hibah, dan internship/magang di industri.
2. Biaya partisipasi dalam pertemuan ilmiah yang diselenggarakan P3FNI.
3. Informasi tentang pangan fungsional dan nutrasetikal Indonesia dan isu internasional terbaru (international current issues).

Iuran Keanggotaan P3FNI

Iuran dari anggota digunakan untuk mendanai kegiatan yang diselenggarakan P3FNI untuk peningkatan keahlian anggota melalui kegiatan ilmiah. Pembayaran menurun progressif 25% jika pembayaran iuran keanggotaan untuk jangka pembayaran 2 tahun sekaligus.

<table>
<thead>
<tr>
<th>Fasilitas</th>
<th>Iuran</th>
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<tbody>
<tr>
<td>Akademisi, Peneliti non-komersial</td>
<td>Rp 400.000</td>
</tr>
<tr>
<td>Praktisi industri, kesehatan, komersial</td>
<td>Rp 500.000</td>
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<tr>
<td>Mahasiswa S2 dan S3</td>
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<td>Mahasiswa S1 (Perlu Rekomendasi)</td>
<td>Bebas Biaya</td>
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Pendaftaran online anggota P3FNI dapat dilakukan dengan masuk melalui web dengan alamat: sia.p3fni.org

Pembayaran dapat dilakukan melalui setor, transfer, pembayaran langsung/cash.

Pembayaran setor dan transfer ditujukan ke no rekening berikut:

**Bank BNI**
Cabang HR MUHAMDAD  
No. rekening 0390796832  
a.n. Indah Epriliati
Journal of Functional Food and Nutraceutical (JFFN) is an official journal of Perhimpunan Penggiat pangan Fungsional dan Nutrasetikal Indonesia or Indonesian Society for Functional Food and Nutraceutical (P3FNI-ISNFF) that was established in collaboration with Research Center for Food and Health, Swiss German University (RC F&H SGU) that published review and research result on the frontier research, development, and application in the scope of functional food and nutraceuticals.