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Extraction, Yield, and Phytochemical Screening of Ethanolic Extract of Red Algae (*Gracilaria gracilis*)

Original Article

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Abstract:
Gracilaria gracilis is a marine red alga (Rhodophyta) rich in bioactive secondary metabolites with promising potential as a pharmaceutical raw material. This study aimed to determine the yield of G. gracilis ethanolic extract obtained via maceration and identify its secondary metabolite profile through qualitative phytochemical screening. Dried *G. gracilis thallus* powder (1,500 g) was extracted using 96% ethanol (1:5 w/v) for 3 × 24 hours with periodic stirring and remaceration, followed by concentration using a rotary vacuum evaporator at 40°C. The concentrated extract was subjected to qualitative phytochemical screening to detect flavonoids, alkaloids, phenolics, steroids, terpenoids, saponins, and tannins. Maceration produced 84.3 g of dark greenish-brown viscous extract, corresponding to an extraction yield of 5.62%. Phytochemical screening confirmed the presence of flavonoids, phenolics, steroids, saponins, and tannins, whereas alkaloids were not detected. These findings demonstrate the efficiency of 96% ethanol as a cross-polarity solvent for extracting diverse bioactive metabolites and establish a fundamental phytochemical baseline supporting the further pharmacological exploration and pharmaceutical product development of G. gracilis.

INTRODUCTION

Marine organisms, particularly macroalgae, are a unique source of bioactive compounds because selective pressures in the marine environment drive the biosynthesis of secondary metabolites with structures and activities rarely found in terrestrial plants (Cikoš et al., 2019). Among the three main divisions of macroalgae, red algae (Rhodophyta) possess the highest species diversity, with over 7,000 species and a rich profile of bioactive compounds including polyphenols, terpenoids, alkaloids, carotenoids, and sulphated polysaccharides making them a priority candidate in the exploration of marine-based pharmaceutical raw materials (Nielsen et al., 2021). *Gracilaria gracilis* belongs to the family *Gracilariaceae* (division *Rhodophyta*) and is widely distributed in the shallow tropical and subtropical waters of Indonesia, with cultivation centres along the coasts of Karawang, Brebes, and Palopo (Murdinah et al., 2013). This species has a cartilaginous cylindrical thallus up to 50 cm in length that grows from an attachment disc (holdfast) in the intertidal zone (Wirawan et al., 2021). The abundant availability of raw material and the ease of its cultivation make *G. gracilis* a promising candidate for development as a source of bioactive pharmaceutical compounds.

Pharmacognosy studies have reported the presence of flavonoids, polyphenols, carotenoids and terpenoids (Purwaningsih and Deskawati, 2021), saponins and steroids (Insani et al., 2022), amino acids (Astorga-España et al.,

2016), fatty acids (El Maghraby and Fakhry, 2015), and essential minerals (Rosemary et al., 2019) in the genus *Gracilaria*. This metabolite profile underpins various documented pharmacological activities: Hakim et al. (2023) demonstrated that red algae gel accelerates mucosal wound healing; Teo et al. (2020) reported synergistic antioxidant, antibacterial and anti-inflammatory effects; and Azzahra and Trimulyono (2024) demonstrated inhibition of pathogenic bacterial growth. Mechanistically, flavonoids, tannins, and saponins act as antiseptics and bacteriostats; steroids inhibit the COX/LOX pathway; whilst tannins accelerate wound epithelialisation (Kusumawardhani et al., 2015).

The success of secondary metabolite extraction depends on the suitability of the method and solvent. Maceration was chosen because its cold extraction principle minimises the risk of degradation of thermolabile compounds such as flavonoids and phenolics (Faiha et al., 2025; Najib, 2018). 96% ethanol was chosen because its semi-polar nature allows for the extraction of both polar and semi-polar compounds in a single step, with a more optimal yield compared to a single solvent (Handoyo, 2020). Qualitative phytochemical screening is an essential preliminary analytical stage for mapping the classes of secondary metabolites to guide the selection of subsequent bioactivity assays. Although the pharmacological activity of various Gracilaria species has been studied, data on the phytochemical profile of *G. gracilis* extracted with 96% ethanol remain very limited. This study aims to: (1) determine the yield of *G. gracilis* ethanol extract via maceration, and (2) identify classes of secondary metabolites through qualitative phytochemical screening.

METHODS

Materials and Equipment

The main material used in this study was fresh *Gracilaria gracilis* thallus obtained from coastal waters [sampling location]. The chemicals used included 96% ethanol (technical grade, for extraction), 95% ethanol (p.a., for flavonoid testing), 2N HCl and concentrated HCl (p.a.), distilled water, magnesium (Mg) powder, iron(III) chloride ($FeCl_3$) solutions 5% and 1%, chloroform (p.a.), anhydrous acetic acid ($C_4H_6O_3$, p.a.), concentrated sulphuric acid (H_2SO_4, p.a.), and Mayer's reagent prepared from a mixture of potassium iodide (KI) and mercury(II) chloride ($HgCl_2$) in distilled water. The equipment used included an analytical balance (accuracy 0.0001 g), a blender, a No. 40 mesh sieve, a glass jar with a lid, a stirring rod, a set of laboratory glassware (beakers, test tubes, Erlenmeyer flasks, measuring cylinders, dropper pipettes), and a rotary vacuum evaporator (equipped with a vacuum pump, water cooler, and temperature-controlled heater).

Preparation of Red Algae Crude Extract

Freshly collected *Gracilaria gracilis* samples are thoroughly washed under running water to remove epiphytes, sediment and organic debris adhering to the surface of the thallus. After washing, the samples are soaked in clean distilled water overnight to soften the tissue matrix whilst leaching out any remaining sea salt and dissolved contaminants that may be present. The drying stage is carried out in the sun for 15 days, with the surface of the samples covered using black cloth; this treatment aims to mitigate direct exposure to ultraviolet radiation, which can degrade photosensitive compounds such as carotenoids and phenolics, whilst maintaining the drying temperature within a safe range (Insani et al., 2022). Once the moisture content of the sample reaches the specified limit, the dried crude drug undergoes dry sorting to separate out non-compliant fragments such as foreign objects, damaged thallus parts, and non-algal material (Liswandari, 2018). The qualified crude drug is then ground using a blender and sieved to obtain a powder with optimal particle size uniformity to maximise the surface area for contact during the extraction process.

Preparation of Red Algae Ethanol Extract

The ethanol extract of *Gracilaria gracilis* was obtained using the maceration method. A total of 1,500 g of dried red algae powder was weighed accurately using an analytical balance, then placed in a sealed glass jar. 7,500 mL of 96% ethanol was added (material:solvent ratio = 1:5 w/v) so that the entire surface of the powder was completely submerged. The 1:5 ratio was selected to optimise the concentration gradient between the solid and liquid phases, thereby maximising the diffusion of active compounds (Afifah et al., 2023). The jar was sealed tightly and stored at room temperature, protected from direct light. Stirring was carried out periodically every three hours using a stirring rod to renew the concentration gradient around the surface of the powder particles and prevent local saturation of the solvent.

The maceration process lasted for 3 × 24 hours. At the end of each 24-hour period, the macerate was filtered using Whatman No. 42 filter paper to separate the filtrate from the residue. The remaining residue was then

remacerated using the same volume of 96% ethanol as in the initial stage; this re-maceration treatment is applied because a solvent that has approached its saturation point will experience a significant reduction in its solubility capacity, so re-maceration helps to maximise the yield of secondary metabolites and increase the final yield percentage (Ningsih et al., 2015). All filtrates from the three maceration cycles were combined, then concentrated using a rotary vacuum evaporator at 40 °C with a rotation speed of 60 rpm until a concentrated, solvent-free extract was obtained. Temperature conditions below the normal boiling point of ethanol (78.4 °C) are made possible by the reduced pressure within the vacuum system, which lowers the solvent's boiling point, allowing ethanol removal without exposing thermolabile compounds to destructive temperatures. The concentrated extract obtained was weighed, and its yield was calculated using the following equation:

Yield (%) = (weight of concentrated extract / weight of dried crude drug) × 100%

Phytochemical Screening of Red Algae Ethanol Extract

A qualitative phytochemical screening was carried out on a concentrated extract of *Gracilaria gracilis* to detect the presence of six major classes of secondary metabolites using validated standard procedures.

The flavonoid test was carried by placing 50 mg of red alga extract is in a test tube, then 1-2 mL of hot water and a small amount of Mg powder are added. Then 4-5 drops of HCl and 95% ethanol are added in equal volumes and shaken. If the mixture turns red, yellow or orange, then the crude extract is positive for flavonoids (Hanapi et al., 2013).

The alkaloid test was carried out by placing a quantity of the extract into a clean test tube, then adding 1 mL of 2N HCl and 6 mL of distilled water. The mixture is heated for two minutes in a boiling water bath to dissolve any alkaloids that may be acid-conjugated under hot conditions, then cooled to room temperature. To the cooled mixture, 2–3 drops of Mayer's reagent are added. Mayer's reagent is a solution of potassium tetraiodomercurate(II) [$K_2(HgI_4)$] prepared by dissolving 1.36 g of $HgCl_2$ in 60 mL of distilled water, then adding a solution of 5 g of KI in 10 mL of distilled water, and diluting the mixture to 100 mL with distilled water. The test result is considered positive if a white to cream-coloured precipitate forms at the bottom of the tube (Nurjanah et al., 2022).

The phenolic compound test was carried out by pipetting 1 mL of extract into a clean test tube, then adding 2 drops of a 5% $FeCl_3$ solution (w/v in 70% ethanol). The tube is gently shaken and the colour change observed over five minutes. The test result is considered positive if a green, dark blue, or black colour forms, indicating the formation of an iron(III)-phenolate complex (Manongko et al., 2020).

The steroid and terpenoid test was carried out using the Liebermann–Burchard reagent. A total of 50 mg of extract was placed in a dry test tube and dissolved in 1 mL of anhydrous chloroform. To this solution, 0.5 mL of anhydrous acetic acid was added, followed by the slow addition of a few drops of concentrated sulphuric acid (H_2SO_4) down the side of the tube to prevent the two layers from mixing immediately. The reaction was observed at the interface between the two liquid layers. The formation of a reddish-brown or violet ring at the boundary of the layers indicates a positive result for terpenoids, whilst the formation of a bluish-green colour indicates a positive result for steroids (Bhayu, 2020). It should be noted that all equipment used in this test must be completely dry, as the Liebermann–Burchard reagent is unstable in the presence of water.

The saponin test was performed by weighing 2 g of extract and placing it into a sealed test tube. Sufficient distilled water is added to fully submerge the sample (approximately 10 mL), then the mixture is heated in a boiling water bath for 2–3 minutes to enhance saponin solubility. The tube is cooled to room temperature, then shaken vigorously vertically for 30 seconds. The tube is left to stand and the height of the foam column is measured after 10 minutes. The test result is considered positive if a persistent (stable) foam forms with a height of ≥1 cm and does not disappear or collapse within ≥10 minutes (Soamole et al., 2018).

The tannin test was carried out by weighing 20 mg of extract and placing it in a test tube. Sufficient 70% ethanol was added to submerge the entire sample, and the mixture was stirred until homogeneous. Two to three drops of a 1% $FeCl_3$ solution (w/v in 70% ethanol) were then added to the solution. The tube is gently shaken and the colour change is observed within five minutes. The test result is considered positive if a black, blue-black or green-black colour forms, indicating the presence of tannin compounds (Soamole et al., 2018).

RESULT AND DISCUSSION

Yield of Red Algae Ethanol Extract

The maceration of *Gracilaria gracilis* using 96% ethanol produced a dark greenish-brown viscous extract with a characteristic seaweed aroma. From 1,500 g of dried crude drug, 84.3 g of viscous extract was obtained, equivalent to a yield of 5.62% (Table 1). Yield is a quantitative parameter that describes the overall efficiency of the extraction process: the higher the yield value, the greater the proportion of metabolites successfully extracted and retained from the initial raw material (Selviana et al., 2024). The yield of 5.62% obtained in this study is substantially higher than that reported by Kurniawati et al. (2016), who obtained a yield of 0.9% from Gracilaria sp. using 96% ethanol as a solvent and the same method.

Variations in yield values between studies on the genus Gracilaria are a common phenomenon and can be attributed to a number of intrinsic and extrinsic factors. Intrinsic factors include genetic variation between species and differences in cell wall composition, whilst extrinsic factors encompass cultivation environmental conditions (salinity, light intensity, water temperature), season and harvest time, as well as the preparation procedure for the crude extract (Delyani et al., 2017). In this study, the use of crude extract powder sieved through a No. 40 mesh sieve contributed positively to the yield, as the uniform and relatively small particle size increased the surface area in contact with the solvent and shortened the diffusion distance of the solute out of the cell matrix (Mohammadi et al., 2021). The semi-polar nature of 96% ethanol also allows for the extraction of both polar compounds (phenolics, flavonoid glycosides) and semi-polar compounds (flavonoid aglycones, terpenoids, steroids) in a single extraction step, resulting in a greater total mass of extracted metabolites compared to using a single polar or non-polar solvent (Handoyo, 2020). Solvent evaporation at 40 °C using a rotary vacuum evaporator has proven effective in minimising the loss of thermolabile compounds during the concentration process, whilst producing a concentrated extract free of solvent residues.

Phytochemical Screening

Phytochemical screening confirmed the presence of flavonoids, phenolics, steroids, saponins and tannins; alkaloids were not detected (Table 1).

Flavonoids. An orange colour following the addition of Mg and concentrated HCl confirms the presence of flavonoids. Mg metal, as a reducing agent, reduces the benzopyrone ring (chromophore) of the flavonoid in an acidic HCl environment, producing a flavilium salt with an extended chromophore that is absorbed in the visible region (Mukhriani et al., 2019). These results are consistent with those of Soamole et al. (2018) on Gracilaria sp., and Ouahabi et al. (2023), who identified catechins, kaempferol, naringin, apigenin, quercetin, and luteolin in G. bursapastoris. Flavonoids act as antioxidants through radical scavenging and metal ion chelation, and have antiseptic effects by disrupting bacterial membrane integrity (Kusumawardhani et al., 2015).

Alkaloids. The absence of a white precipitate confirms that no alkaloids were detected. Mayer's reagent [$K_2(HgI_4)$] acts by forming a coordination complex between Hg^{2+} ions (Lewis acid) and the lone pair of electrons on the nitrogen atom of the alkaloid (Lewis base), resulting in a potassium-mercury-alkaloid complex precipitate (Harahap, 2023). This negative result is consistent with Bhernama (2020) on Gracilaria sp. The absence of alkaloids is likely due to very low natural content in this species and/or the degradation

Table 1. Results of phytochemical screening of *Gracilaria gracilis* ethanol extract

Compound Group	Reagent	Result	Reaction Notes
Flavonoids	Mg powder + concentrated HCl	(+)	Orange colour
Alkaloids	Mayer's reagent	(–)	No white precipitate
Phenolic	$FeCl_3$ 5%	(+)	Blackish-green colour
Steroid	Anhydrous acetic acid + H_2SO_4	(+)	Blue-green colour
Saponin	Distilled water (foam test)	(+)	Stable foam ≥10 minutes
Tannin	Ethanol + 1% $FeCl_3$	(+)	Blackish-green colour

of heterocyclic nitrogen structures due to photooxidation during sun-drying (Lantah et al., 2017).

Phenolics. A blackish-green colour following the addition of 5% $FeCl_3$ confirmed the presence of phenolics. Fe^{3+} ions form chelate complexes with phenolate groups ($-O^-$) via the acceptance of oxygen's lone pair of electrons, producing chromophore iron-phenolate complexes; the colour shade depends on the number of -OH groups and the structural complexity of the compound (Kurang et al., 2020). Ouahabi (2023) identified nine phenolic acids in G. bursa-pastoris, including gallic acid, caffeic acid, and chlorogenic acid. Phenolics act as primary antioxidants through the donation of hydrogen atoms to free radicals and as antimicrobials by disrupting cell wall synthesis (Kusumawardhani et al., 2015).

Steroids. A bluish-green colour confirms the presence of steroids. The Liebermann–Burchard reagent operates in two stages: anhydrous acetic acid acetylates the steroid -OH group at C-3 under anhydrous conditions, followed by concentrated H_2SO_4 catalysing the dehydration and oxidation of the steroid skeleton to form a conjugated polyene system as the visible chromophore (Anggraini and Nabillah, 2018). These results are consistent with those of Soamole et al. (2018) on Gracilaria sp., and the report by Hakim et al. (2023), which found 7.40% cholesterol in G. verrucosa. Red algal steroids possess anti-inflammatory activity through the inhibition of the COX and LOX pathways, suppressing the biosynthesis of prostaglandins and leukotrienes as mediators of pain and inflammation (Kusumawardhani et al., 2015).

Saponins. A stable foam of approximately 1 cm that persists for ≥10 minutes confirms the presence of saponins. Saponins are amphiphilic because they possess a lipophilic aglycone core (sapogenin) conjugated with a hydrophilic oligosaccharide chain. When shaken, saponins orient themselves at the air-water interface, reducing surface tension and stabilising bubbles through steric effects, thereby preventing the foam from collapsing easily (Putri et al., 2023). These results are consistent with those of Aprinaldi (2020) on G. verrucosa. Saponins act as antiseptics by intercalating sapogenin into the lipid bilayer of the microorganism's cell membrane, increasing membrane permeability, and causing lysis (Kusumawardhani et al., 2015).

Tannins. The blackish-green colour following the addition of 1% $FeCl_3$ confirms the presence of tannins. A colour that is significantly darker than that observed in standard phenolic tests is characteristic of tannins: the abundance of free -OH groups per molecule allows the formation of polydentate chelate complexes with multiple Fe^{3+} ions simultaneously, resulting in iron-tannin polymers with highly extensive chromophores that absorb almost the entire visible spectrum (Taminggu and Tahril, 2022). These results are consistent with those of Maftuch et al. (2016) on *G. verrucosa*. Tannins exert an astringent effect through cross-linking with collagen proteins, promoting tissue contraction and forming a protective wound layer; simultaneously, they function as antimicrobials and antioxidants that accelerate epithelialisation (Kusumawardhani et al., 2015; Taminggu and Tahril, 2022).

Overall, the combination of identified flavonoids, phenolics, steroids, saponins, and tannins forms a synergistic bioactive matrix that provides a strong scientific basis for more targeted research into the quantitative bioactivity and isolation of specific active compounds from *G. gracilis*.

CONCLUSION

Extraction of *Gracilaria gracilis* using the maceration method with 96% ethanol (1:5 w/v, 3 × 24 hours) yielded a yield of 5.62% (84.3 g from 1,500 g of dried crude drug). Phytochemical screening confirmed the presence of flavonoids, phenolics, steroids, saponins and tannins, whilst alkaloids were not detected. The diversity of these secondary metabolites demonstrates the effectiveness of 96% ethanol as a cross-polarity solvent, whilst providing a phytochemical baseline for *G. gracilis* that justifies its pharmacological potential as a foundation for further bioactivity research and pharmaceutical product development.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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