The Potency of Rhizophora mucronata Leaf Extract as Antidiarrhea

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ABSTRACT

Rhizophora mucronata is commonly known as mangrove growth in the tropical and subtropical region coastlines. It is a natural source of tannins and flavonoid. R. mucronata leaf has been used in the folk medicine for treating diarrhea or gastric motility disorder. The aim of the study was to evaluate the effect of R. mucronata leaf extract on ileum motility in rats. This study was intended to identify active compound from R. mucronata leaf by phytochemical screening. The characterization of tannins from R. mucronata leaf used reversed phase high performance liquid chromatography (HPLC). Crude extract of leaf was evaluated on isolated rat ileum. Anti-motility effect of different concentrations (0.15-0.75%) of R. mucronata leaf extract was assessed on methacholine precontracted isolated ileum. An isolated ileum of rat was immersed in an organ bath which contained aerated Tyrode solution. The tissue was stimulated with methacholine (10^-5M) and its responses were recorded by Power Lab. Various concentrations of the extract (0.15%, 0.30%, 0.45%, 0.60% and, 0.75%) were applied to the tissue then the tissue’s responses were recorded. The result showed that the extract relaxed or reduced the contractions of isolated rats ileum. Based on the High Performance Liquid Chromatography (HPLC) analysis, R. mucronata leaf extract contained catechin 47.428 ppm and epigallocatechin 3.150 ppm. The result indicated that the components of R. mucronata leaf had a potential use in the treatment of gastrointestinal motility such as diarrhea. The most effective concentration of R. mucronata leaf extract in decreasing motility of the ileum was 0.50%, reduced 51.52 (%) ± 10.85 motility of the ileum by direct action on smooth muscle cells.

Key words: Rhizophora mucronata, condensed tannins, diarrhea, ileum motility

Introduction

Marine environment continuously provides a wealth of organisms with structurally unique bioactive secondary metabolites (Sennet et al., 2002). Many marine organisms are medicinal resources e.g macroalgae, microalgae, sponges and mangrove.

Mangrove has been used as traditional medicine in South Asian countries. Recently scientists are veering in search of effective remedies from mangroves for diseases such as diabetes, asthma, cancer, ulcer, wounds and AIDS (Premanathan et al., 1999; Babu et al., 2001; Itigawa et al., 2001).

Two basic factors justify the study of the chemical constituents of mangrove plants. Firstly, mangroves are one of the easiest tropical forest types to generate. The second reason is that numerous mangrove plants have been used in folklore medicine, and recently, extracts from mangroves and mangrove-dependent species have proven activity against human, animal and plant pathogens but only limited investigations have been carried out to identify the metabolites responsible for their bioactivities (Bandaranayake, 2002).

Rhizophora mucronata is commonly known as mangrove growth in the tropical and subtropical region coastlines (Rohini and Das, 2009). R. mucronata leaf has been used in the folk medicine for treating diarrhea or gastric motility disorder (Bandaranayake, 1999). In East Java, mangrove societies boil R. mucronata leaf for diarrhea therapy. In ethno medicine, the R. mucronata bark is also mentioned for its anti diarrheal properties. There is no significant report on the anti diarrheal activity from leaf before. Diarrhea is characterized by increased frequency of bowel movement, wet stool and abdominal pain. Neurohormonal mechanisms, pathogens, malnutrition, chronic disease and drugs can alter gastrointestinal physiology resulting in changes in either secretion or absorption of fluid by the intestinal epithelium. Anti-motility compounds such as diphenoxylate, anticholinergic have been used to treat against diarrheal disorders but they often come with side effect after prolonged use (Harrison, 2005).

Anti diarrheal properties of medicinal plants have been known to relate with the presence of tannins and flavonoid. According to Rohini et al. (1999) R. mucronata leaf is a natural source for both tannins and...
flavonoid, but their chemical, biological and pharmacological properties have not yet been determined (Rahim et al., 2008).

Tannins are classified into hydrolysable and condensed tannins (CT). Hydrolysable tannins (HT) represent polyesters of gallic acids (gallotannins) and hexahydroxydiphenic acids (ellagitannins) (Haslam, 1998). Condensed tannins (CT) or proanthocyanidins (PA) refer to complex of oligomers and polymers of flavonoid units (Haslam (1998); Schoefield et al. (2001)). There are no reports the kind of tannins in R. mucronata leaf.

The aim of the study was to evaluate the effect of R. mucronata leaf extract on ileum motility in rats. This study was intended to identify active compound from R. mucronata leaf by phytochemical screening. The characterization of tannins from R. mucronata leaf was done by using reversed phase high performance liquid chromatography (HPLC).

Materials and Methods

Plant Collection and Preparation of the Extract

Fresh samples of the R. mucronata mangrove leaves were collected in July, 2009 from Probolinggo, East Java. The leaves of R. mucronata were air-dried in the laboratory, grounded into fine powder. It was extracted with methanol using maceration method. The crude methanol extract obtained was concentrated in vacuum. It was then properly labeled and stored in the refrigerator at 4°C until it was used.

Phytochemical screening

The crude extracts were used for preliminary screening of phytochemicals such as alkaloids (Mayer and Dragendorff’s tests), flavonoids (Mg tests), saponins (foam tests), sterols and triterpen (Liberman-Burchard, and Salkowski tests), tannins (FeCl₃ and gelatin test).

High Performance Liquid Chromatography (HPLC) analysis

All chromatographic solvent are HPLC grade. The crude methanol R. mucronata leaf extract was further extracted with chloroform followed by ethyl acetate. The leaf extract was dried (70°C) in water bath and used N₂. The dry sample filtrated through membrane filter with an aperture size of 0.45 µm. The crude methanol R. mucronata leaf extract was analyzed by HPLC. The mobile phase consists of H₃PO₄ 0.05% and acetonitrile. Sample was eluted at a flow rate of 1 ml/min and detected by UV-Vis at 275 nm wave length. Catechin and epigallocatechin gallat performed as the standards.

In Vitro Effect on Isolated Rat Ileum

Rats were supplied by Pharmacology Laboratory of Medicine Faculty, Brawijaya University. An adult rat, which had free access to water but starved overnight prior to the experiment, was used. Rat was anesthetized with Chloroform. A segment (1-2 cm) of the terminal ileum was removed. Each segment was washed to remove any remaining food material with Tyrode solution. The segment was immersed in Tyrode solution. It was set up in an organ bath filled with warm (37°C) aerated Tyrode solution. The segment of ileum was connected to an isotonic transducer (load 1 g) connected to a Power Lab data system. The ileum was stimulated with methacholine (10⁻⁴-10⁻⁷M) in order to obtain a maximal contraction (80% contraction). Based on the Power Lab data, concentration of methacholine 10⁻⁵M gave maximal contraction. At the beginning of each experiment, the concentration was used to stimulate contraction. The crude methanol R. mucronata leaf extract was added to chambers in some cumulative concentrations (0.15%, 0.30%, 0.45%, 0.60% and, 0.75%). The response of each administration of the extract was recorded until showing stability. It is expressed by the percentage of the initial contractions.

Results and Discussion

Identification of active compound from R. mucronata leaf

The phytochemical content of R. mucronata is shown in Table 1. Phytochemical screening revealed the presence of tannins, alkaloid, flavonoid, terpenoid and saponin on the crude methanol R. mucronata leaf extract. Ferric chloride was used to indicate the presence of condensed tannins and gallic tannins. A blackish blue color indicated the presence of gallic tannins while green blackish color, indicated the presence of condensed tannins (Indrayani et al., 2003). R. mucronata leaf contains flavonol and flavanon. Based on the ferric chloride test,
crude methanol extract R. mucronata leaf contained condensed tannins, not hydrolyzed tannins. Then, to identify condensed tannin content, analysis was further done using HPLC.

According to Rohini et al., (1999) R. mucronata leaf is a natural source of tannins and flavonoid. Tannins are phenolic plant secondary metabolites and are involved in plant–pathogen (Brownlee et al., 1990; Heil et al., 2002). Flavonoids are among the most ubiquitous groups of plant secondary metabolites distributed in various foods and medicinal plants.

Table 1: Phytochemical Screening of R. mucronata leaves extract

<table>
<thead>
<tr>
<th>Phytochemical</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>+</td>
</tr>
<tr>
<td>Sterol</td>
<td>-</td>
</tr>
<tr>
<td>Terpenoid</td>
<td>+</td>
</tr>
<tr>
<td>Tannin</td>
<td>+</td>
</tr>
<tr>
<td>Saponin</td>
<td>+</td>
</tr>
</tbody>
</table>

- : not detected; + : detected

CT refers to a complex of oligomers and polymers of flavonoid units. Catechin is a major component of the condensed tannin. Principal catechin includes epicatechin, epicatechin-3-gallate, epigallocatechin, and epigallocatechin-3-gallate (EGCg) (Ceregrzyn and Kuwahara, 2003). In the present study, we used HPLC to characterize CT. In HPLC analysis, we used catechin and epigallocatechin gallate as chemical marker. The HPLC analysis showed the active compounds of crude methanol extract of R. mucronata are catechin and epigallocatechin gallat (Fig.1). The dominant CT is catechin (47.43 ppm) and followed by epigallocatechin gallat (3,150 ppm). In addition, the KEGG BRITE Database (2010) grouped both of them into flavonoid.

Fig. 1: HPLC chromatogram (275nm) of crude methanol extract of R. mucronata leaf.

Table 2: Identification of R. mucronata leaves extract by HPLC

<table>
<thead>
<tr>
<th>Peak</th>
<th>Retention Time</th>
<th>Condensed Tannin</th>
<th>Area</th>
<th>Concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3.620</td>
<td>Catechin(*)</td>
<td>844.530</td>
<td>10.000</td>
</tr>
<tr>
<td>4</td>
<td>4.913</td>
<td>Epigallocatechin gallat(*)</td>
<td>27.531</td>
<td>0.250</td>
</tr>
<tr>
<td>9</td>
<td>3.508</td>
<td>Catechin( ** )</td>
<td>1.971.4</td>
<td>23.624</td>
</tr>
<tr>
<td>13</td>
<td>4.917</td>
<td>Epigallocatechin gallat( ** )</td>
<td>126.026</td>
<td>1.575</td>
</tr>
</tbody>
</table>

(*) standard solution; (**) R. mucronata leaves extract

Based on the identification by HPLC showed that R. mucronata leaves extract have same retention time with standard solution (catechin and epigallocatechin gallat). R. mucronata leaves extract contains catechin and epigallocatechin gallat. According to the chromatogram in Figure 1, catechin and epigallocatechin gallat appear on the peak 9 and 13. Crude leaves extract contains catechin 47.428 ppm and epigallocatechin 3,150 ppm.

Both of catechin and epigallocatechin gallat is grouped as condensed tannins. CT are oligomers of flavan-3-ol monomers such as catechin or epicatechin and flavon 3,4-diols (leucoanthocyanidins) that form the ‘starter unit’ on which tannin condensation takes place (Paolocci et al., 2004). Based on the KEGG Brite Database, catechin an epigallocatechin gallat form into proanthocyanidin group. Proanthocyanidin is the part of flavonoid.

Flavonoid biosynthesis pathway is a combination of shikimic acid pathway and acylpolyolacat. Derivate of cinnamic acid (phenilprophan), synthesized from shikimic acid, act as precursors in the synthesis of polyketides, followed by the addition of three acetate residues. Polyketide formation is then followed by a
closed ring. Through a series of hydroxylation and reduction, plants synthesize a variety of flavonoids (Bravo and Mateus, 2008).

Tannins are secondary metabolites. In general, secondary metabolites are stored in the vacuole. R. mucronata leaf has idioblast cells. Idioblast cell is the cell that contains the product of secondary metabolites. Tannins in Rhizophora sp leaves located in the idioblast cell. Inside cells, the tannins are in the vacuole. Idioblast cells containing tannins are located in the arenchimathos (Kathiresan and Bingham, 2001). Increasing tannin content at saline conditions and/or early growth stages suggested that there is a defense mechanism to cope with environmental stresses (Badi and Sorooshzadeh, 2011).

Catechin is active compound in green tea. Catechins affect diverse functions of the digestive tract, including gastrointestinal tract motility and transit, while epigallocatechin gallate myenterik affect neuronal depolarization in guinea pig small intestine (Ceregrzyn and Kuwahara, 2003). Referring to the green tea compound and their function, therefore R. mucronata which contains catechin and epigallocatechin gallate, can be used as a diarrhea medicine, as has been done by the community in the mangrove region.

Isolated Rat Ileum In Vitro:

A variety of gastrointestinal (GI) disorders, including colonic pseudo obstruction and chronic constipation, are related to impaired GI motility and transport (Jalilian et al., 2004). Diarrhea is caused by hypermotility. Drugs that have effect on the motility or propulsion rate of the gastrointestinal tract may have anti-diarrhea effect (Sunday et al., 2008). In mild diarrheas, anti-motility agents will lessen stool frequency. And by increasing the time of contact with the gut epithelium will also lessen stool volume (Field, 2003).

The studies in isolated organ are considered as a useful technique to evaluate the effect of medicine. Methacholine increases tonus contraction amplitude and peristaltic activity of stomach and intestine (Brown and Taylor, 2003). On the previous exploration, methacholine which gives 80% contraction is concentrated at 10^{-5}M. Methacholine as stimulator is more stable than acetylcholine is. Methacholine that is hydrolyzed slowly by cholinesterase has longer duration action. The result showed that the extract significantly relaxed the isolated rats ileum and reduced their contractions induced by methacholine (10^{-5}M), (Table 3.).

<table>
<thead>
<tr>
<th>Extract Concentration (%)</th>
<th>Relaxation Effect (%) (Means ±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (0.15)</td>
<td>24.85 ± 3.05</td>
</tr>
<tr>
<td>B (0.30)</td>
<td>51.52 ± 10.85</td>
</tr>
<tr>
<td>C (0.45)</td>
<td>65.27 ± 15.03</td>
</tr>
<tr>
<td>D (0.60)</td>
<td>67.67 ± 26.54</td>
</tr>
<tr>
<td>E (0.75)</td>
<td>76.71 ± 27.00</td>
</tr>
</tbody>
</table>

*Statistical was performed using analysis of variance (ANOVA) followed by Post Hoc test (P<0.05)
Values on the columns with different superscripts showed significantly different (P<0.05)

A significant effect of crude methanol extracts of R. mucronata leaf was observed at concentration 0.30% (p<0.05) (Tab.1). The final concentration of the extract (0.30 %) almost completely inhibited the ileum contraction (51.52 %). There was a significant correlation between concentrations of extract and their relaxation effect (r = 0.733). All the concentration extracts significantly inhibited methacholine induced contraction as compared with the control (base line) (Fig 2).
In this study, the crude methanol extract of *R. mucronata* leaf relaxed spontaneously contracting rat ileum after transient contraction. Methacholine acts mainly on muscarinic M3 receptor on circular smooth muscle cells. Contraction of all smooth muscles, including those of gastrointestinal tract, absolutely depends on the presence of Ca\(^{2+}\). Agonist-induced contractions may be related to the release of intracellular Ca\(^{2+}\) from sarcoplasmic stores in addition to the influx, mainly through L-type Ca\(^{2+}\) channels of extracellular Ca\(^{2+}\). (Makhlouf, 1994). The blockage of the calcium channels would result in reduced influx of calcium ions into the sarcoplasmic reticulum. Thus it causes a reduction in cytosolic calcium ion concentration which in turn causes a reduced binding of calcium to the protein calmodulin. The calcium-calmodulin complex should activate myosin light chain kinase (MLCK) with the resultant phosphorylation of the light chains. If such phosphorylation occurs, interaction between actin and myosin is resulting in smooth muscle contraction (Gilani, et al., 2005).

Hence inhibition of calcium will result in a break in the cascade producing relaxation. The binding of methacholine and muscarinic receptor initiates multiple signaling pathways that induce Ca\(^{2+}\) influx, and stimulate phospholipase C (PLC). PLC hydrolysis phosphatidyl inositol 4,5-biphosphate (PIP\(_2\)). The result of PIP\(_2\) hydrolysis consists of inositol 1,4,5-triphosphate (IP\(_3\)) and dycylglycerol (DAG). The spasmyloytic constituents of various plants are mediated through blockage of calcium channels (Gilani, et al., 2005). The total relaxation produced the extract at concentration 0.30 % (Fig-2).

Crude methanol extract of *R. mucronata* leaf directly inhibits intestinal motility with a mechanism involving L-type Ca\(^{2+}\) channels. It blocks metacholin at muscarinic receptor. This bound inhibit release IP\(_3\), and Ca\(^{2+}\) into the cells. Finally, this reaction inhibits smooth muscle contraction (Katzung, 2001). The crude methanol of *R. mucronata* leaf extract has potency to relax smooth muscles contraction and to reduce ileum motility of rats by using anticholinergic mechanism. It is well known that drugs which inhibit intestinal transit in patophysiologic state may be effective in alleviating diarrhea (Borelli, et al., 2006).

Anti diarrheal properties of medicinal plants are related with phytochemical compounds such as tannins and flavonoids. Catechin influenced gastrointestinal function other than motility. Epigalocatechin gallat derived from leaves can alter spontaneous activity of the small intestine (Ceregryzyn and Kuwahara, 2003). Potency of relaxation from the extract related with flavonoid content. This effect related to interference with calcium influx and calcium released from intracellular stores. The therapy of anti-diarrhea crude methanol extract of *R. mucronata* leaf is symptomatic therapy. Crude methanol extract of *R. mucronata* leaf press intestinal peristaltic. In this condition, cholinergic effect was dominant. Thus, crude methanol extract of *R. mucronata* leaf reduced cholinergic effect with press intestinal peristaltic because it contains catechin and epigalocatechin gallat. The results of this study also support the use of *R. mucronata* leaf in ethno medicine for gastrointestinal disorders and diarrhea.

**Conclusion:**

1. Phytochemical screening revealed the presence of tannins, alkaloid, flavonoid, terpenoid and saponin on the crude methanol *R. mucronata* leaf extract. The type of tannins in crude methanol extract of *R. mucronata* leaf is condensed tannin. It contained catechin 47.428 ppm and epigallocatechin 3.150 ppm.

2. Crude methanol extract of *R. mucronata* leaf is used in the treatment of gastrointestinal motility such as diarrhea. It inhibits the motility of the ileum by direct action on smooth muscle cell and act as antagonist cholinergic receptor.

3. A significant effect of crude methanol extract *R. mucronata* leaf was observed at concentration 0.30%. This concentration relaxed 51, 52 % ileum contractions induced by methacholine (10^{-M}).

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Inhibition of Gastric Emptying and Intestinal Motility by Fractions of *Maytenus ilicifolia* Mart ex. Reissek.


