Induction of Apoptosis and Antiangiogenesis

Effects of Pinostrobin from *Kaempferia pandurata* Roxb against Induction of Fibrosarcoma Mice

Results Benzopiren

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Abstract

Induction of apoptosis and antiangiogenesis effects of Pinostrobin from *Kaempferia pandurata* Roxb against Fibrosarkoma mice results benzopiren induction has been done. Examination or surgery begins taking tissue fibrosarcoma in mice infected and weigh fibrosarcoma obtained. Fibrosarcoma tissues were then stored in containers that have contained 10 % formalin. Weighing results showed that the concentration of pinostrobin oral 80 mg / kg can inhibit the growth of fibrosarcoma with a gram weight of 68.62 % and a cancer drug (control + ) there is resistance 95.95 % compared to the negative control which is only given orally CMC - Na, this means pinostrobin potentially be developed as a cancer chemotherapy.
Furthermore done shooting patohistologi tissue fibrosarcoma with HE staining with a light microscope with 400x magnification. The results obtained showed many chromatin (polikromatin) which prove the damage caused by having fibrosarcoma cells.

Immunohistochemical assay showed oral pinostrobin concentration 80 mg / kg body weight can increase the expression of p53 to apoptosis induction could take place and the decreased expression of VEGF angiogenesis which proves the existence of barriers.

**Keywords:** Pinostrobin, fibrosarcoma, apoptosis, antiangiogenesis, p53 dan VEGF

**Introduction**

Rhizome plants of *Kaempferia pandurata* Roxb as a traditional medicine in Indonesia in general is widely used as a dry cough, cancer sores, irritation of the colon, stomach bloated, difficult urination in children, inflammation of mucous membranes in the mouth of the womb, dysentery, and tumor / cancer. Rhizomes of *Kaempferia pandurata* Roxb was extracted with n-hexane produce levels of flavonoid components pinostrobin relatively large, namely 2.5% and as much as ±20 grams / 800 grams of powder or alpinetin approximately 1% (Oka, 2000). The results of the study as it turns out pinostrobin have anti-oxidant activity and relaxes smooth muscle. One method to test materials - materials that are cytotoxic are the toxicity tests on larvae shrimp (*Artemia salina* L.). This method is often used for initial screening of the active compounds as anti-cancer agent in the extract of the plant because they are easy, inexpensive, fast and reliable outcome (Oka, 2000)

The content of rhizome pinostrobin in *Kaempferia pandurata* Roxb is big enough then pinostrobin isolation as a pure substance can be done fairly quickly. Pinostrobin the polarity of the structure is reduced as a result of intra-molecular hydrogen bonding between the carbonyl group at C-4 with a hydroxy group at C-5 position. Then extraction can be performed with less polar solvents such as chloroform and n-hexane (Oka, 1998)

It can be seen in the structure below:

![Pinostrobin](image)

![Alpinetin](image)

![Pinocembrin](image)

Based on the structure then pinostrobin can be identified by UV-Vis spectroscopy to look at λ max as a characteristic of flavonoids consists of two absorption bands are bands I (325 nm) and II bands (287 nm) and the bathochromic shift when coupled shear reaction AlCl3 at 20 -26 nm to indicate the presence of-OH substituent at C-5 position. Identification by IR spectroscopy
to look at group functions, Proton and Carbon NMR spectroscopy to look at the type and amount of the H and C atoms of Pinostrobin and mass spectroscopy to look at relative molecular mass (Mr) and fragmentation of 5-hydroxy-7-methoxy flavanones or pinostrobin (Markam KR, 1988; Silverstein, Bassler and Morrill, 1981).

Pinostrobin flavonoid compounds have been isolated from the rhizome of *Kaempferia pandurata* Roxb with levels of ± 2.5%, and it is known that pinostrobin compounds have activity inhibiting the growth of human breast cancer cells and inhibit the enzyme activity of DNA Topoisomerase I (Oka, 1998). DNA Topoisomerase enzymes have an important function in intracellular processes, which play a role in the replication process and the proliferation of cancer cells. By being such that the enzyme activity by DNA Topoisomerase inhibitor compounds, the process of bonding between the enzyme with the longer DNA of cancer cells. So that will be formed Protein Linked DNA Breaks (PLDB), resulting in cancer cell DNA fragmentation and the subsequent effect on the cells in the process, especially the process of cell replication, which ended with the death of cancer cells (Lowe Scoot W., Copero gerard Enrique and Evan, 2004; Sukardiman et al., 2006).

The presence of DNA damage and subsequent cell cancer affects the cells in the process, especially the process of cell replication ends with the death of the cancer cell apoptosis (Sukardiman et al., 2006). Thus compounds suspected pinostrobin have cancer chemopreventive activity and therapeutic in vivo in mice results Fibrosarkoma cancer induction Benzopirena. The presence of DNA damage allegedly cells can activate the p53 tumor suppressor gene can induce apoptosis in addition, can also affect the cell cycle by affecting the cell cycle inhibitor p27 and cyclin-dependent enzymes barriers kinase (C. Whibley, 2009).

The presence of p53 activation and stabilization products by pinostrobin then be expected to inhibit angiogenesis through the mechanism of the effect of down-regulation or decreased expression of VEGF (Vascular Ephidermal Growth Factor) and decreased expression of Cyclooxygenase-2 (COX-2) and matrix metalloproteinase - 9 (MMP-9) were also involved in the metastasis of cancer cells. Angiogenesis is the formation of blood capillaries of solid cancer cells that have a size larger than 1-2 mm, the result would be the development and growth of cancer and may eventually spread throughout the network (C. Whibley, 2009; Samiasih S., 2010; Chrestella J., 2009).

**Materials and Methods**

The material used is the rhizome powder *Kaempferia pandurata* Roxb were obtained from Badung Market, Denpasar, Bali. Mice used were male mice, aged 2 months and weigh 20-25 grams.

The chemicals used are technical hexane, n-hexane (pa), methanol (pa), ethanol (pa), chloroform (pa), ethyl acetate (pa) distilled water, silica gel and TLC plates with silica gel GF Aluminum-254 from E Merck), primary and secondary antibodies p53 and VEGF 10% formalin buffer, PBS, DAB, H2O2 3% and Destilate Water (DW).
The tools used are HPLC and TLC-densitometer Shimadzu CS-930, UV-Vis Spectrophotometer Hitachi 557 and 365 Shimadzu NIR, IR spectrophotometer Jasco 5300 FTIR, NMR spectrometers Proton and Carbon NMR Hitachi FT-1500 and HP 8890 Mass Spectrometer, Light Microscopy, polilysin preparations and glass objects.

**Isolation of Pinostrobin on Rhizome of *Kaempferia pandurata* Roxb)**

Weighed 1000 grams powdered rhizome key retrieval, then do percolation with n-hexane for 24 hours (1 day), which is accommodated perkolat evaporated with rotary evaporator. N-hexane extract allowed to arise crystalline amorphous. Amorphous crystalline subsequent vacuum column chromatographed with n-hexane developers. Results of column chromatography and then accommodated, then back to rotary evaporated to arise evaporator crystal. The crystals filtered, then recrystallized with methanol 3 times. Crystals were obtained shiny white. The crystals obtained are then determined by DTA melting point and Melting Points Apparatus Fiesher & John and tested purity by TLC with several different eluent polarity, TLC - densitometer with multiple wavelengths and HPLC. The purity of the test results can be said to be pure if pinostrobin \( \lambda \) subsequently identified structures with UV - Vis Spectroscopy to see max and shift batokromiknya, IR function to determine cluster, Proton and Carbon NMR spectroscopy to determine the type and amount of H and C atoms and mass spectroscopy to determine the relative molecular mass of 5-hydroxy-7-methoxy flavanones / pinostrobin.

**Induction of cancer cell Fibrosarkoma Induction in Mice Results Benzopirenna**

Fourty male mice tails undergo a process of adaptation studies, all mice were subsequently induced benzopirenget as much as 0.3 mg / 0.2 mL in oleum olivarium subcutaneous injection in the scapular region 5 times, every other day. Subsequently, the whole atmosphere and maintained in mice the same diet for two months / form of cancer in the nape area, after reaching the cancer volume ± 100 mm\(^3\), mice with cancer were randomly divided into 3 groups. Group I is the negative control, only given CMC-Na. Group II was given pinostrobin dose of 80 mg / kg; Group III was given cyclophosphamide at a dose of 13.33 mg / kg. All test materials are given in intraperitonial (oral) and administered daily for 14 days. After the mice were sacrificed and done taking the cancerous tissue and then do the weighing of cancer.

**Examination of expression of p53 and VEGF expression in Immunohisto-Chemistry**

At first conducted clearing and rehydration in stages against both cancer slice preparations both the control group and the treatment group were stored in 10 % formadehid solution, then washed 3 times with destilate water (DW), respe-
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Actively 5 minutes then drained fluid around the network. After that the tissue pieces circled with a pen and then immersed in 3 % H₂O₂ in DW 15 minutes at room temperature. Then the preparations were rinsed 3 times with DW and 3 times with PBS each 5 minutes.

The next step preparations were incubated with 10 % normal goat serum 30 min at room temperature and then rinsed 3 times with PBS each 5 minutes. Then the preparations were incubated with primary and then rinsed 3 times with PBS each 5 minutes, after which it was incubated with the secondary for 30 min at room temperature. Avidin and biotin were incubated in 1 ml of PBS, μ I 10 μ At the same time, 10 Furthermore, preparations were rinsed 3 times with PBS each 5 minutes and then incubated with a mixture of avidin and biotin 30 min at room temperature and then rinsed 3 times with PBS 5 min each and incubated with DAB solution. The next step performed counterstaining with hematoxylin and then dehydrated, then covered with a cover glass preparations. Mixture further examined by microscope to see the expression of p53 and VEGF expression. Penbacaan done with 400x magnification are seeing positive and negative.

Results and Discussion

Isolation and Identification Pinostrobin

Positive isolates flavonoids are flavanones sample solution when coupled with FeCl₃ in methanol, the solution became red, plus concentrated HCl and Mg powder, the color of the solution became pink and added 10 % NaOH, the solution became light yellow. Test purity by TLC using the mobile phase: n - butanol : acetic acid : water = 4:1:5 ; chloroform : methanol = 3:1 Chloroform : Ethanol = 15:1, n - hexane : ethyl acetate = 1:1, n - hexane : ethyl acetate = 3:1, n - hexane : ethyl acetate = 3:2 and n - hexane : ethyl acetate = 7:3, after eluasi finished plate sprayed or placed on ammonia vapor and observed under UV light 254 nm obtained a stain. Test purity by TLC - densitometry at a wavelength [220 nm (I), 254 nm (II), 287 nm (III), 300 nm (IV), 320 nm (V), and 350 nm (VI)] obtained a peak and purity was tested by HPLC purity obtained 5 - hydroxy - 7 - methoxy flavanones isolated 94.83 %. The purity of the test results it can be said that the 5-hydroxy-7-methoxy flavanones isolated pure by Chromatography assay (Silverstein, Bassler and Morrill, 1981, Oka, 1998).

Analysis of the melting point Melting Points Apparatus Feisher & John obtained melting point range = 99 - 100°C (I); 99.5 to 101.5°C (II) and from 99.5 to 100.5°C (III) and the obtained DTA melting point = 97°C and the stability of the substance to a temperature of 150°C. Identification max = 287 nm (bands II) and λ by UV-Vis spectrophotometer obtained shoulder = 325 nm (tape I), which is in line with the literature in which flavonoids flavanones when analyzed by UV-Vis spectrophotometer will show two absorption bands are between 275 - 295 nm (bands II) and a shoulder at 300-330 nm due sinamoil and benzoyl group of flavanones, which then when pinostrobin solution in methanol is added to the AlCl₃ absorption band II bathochromic -shifted by 23 nm, indicating that a pino-
strobin flavonoids flavanones containing OH at C-5, as shown in the structure below (Silverstein, Bassler and Morrill, 1981, Oka, 1998).

Identification by IR spectroscopy obtained wave numbers 3437.46 cm\(^{-1}\) (-OH indicate the hydrogen bonding with the -C=O at C-4); 3061.31 cm\(^{-1}\) (show -CH stretch of aromatic), 1645, 43 cm\(^{-1}\) (-C=O shows that ketones undergo hydrogen bonding with the -OH at C-5), 1622.26 and 1579.64 cm\(^{-1}\) (-C=C show-aromatic); 3000-2800 and 1450-1350 cm\(^{-1}\) (show -methyl and methylene CH), 1250 and 1290 cm\(^{-1}\) (show -CO) (Silverstein, Bassler and Morrill, 1981, Oka, 1998).

Proton NMR spectroscopy resulted in chemical shift (\(\delta\)) at 3.75 ppm, indicates ArOCH\(_3\) (3H), 6.019 ppm (singlet) shows the H-6 and H-8 (2H); 11.948 ppm (singlet) showed -OH (1H); 2.817 to 3.029 ppm (multiplet) shows the H-3 cis or trans (2H), multiplets in this case due to the overlap and the different constants of H -trailer that is in cis or trans position; 5.284 ppm (dd) shows the H-2 (1H) while for the Carbon NMR chemical shifts in the produce 55.583 ppm indicates O-CH\(_3\) (1C); 79.121 ppm showed C-2 (1C); 43.326 ppm showed C-3 (1C); 195.440 ppm showed C-4 (1C); 163.944 ppm showed the C-5 (1C); 95.037 ppm indicates the C-6 (1C); 167.755 ppm showed the C-7 (1C); 94.153 ppm showed C-8 (1C), 162.572 ppm showed the C-9 (1C); 103.025 ppm showed C-10 (1C); 138.241 ppm showed C1' (1C); 125.954 ppm showed C2' dan C6' (2C); 128.667 ppm shows C3'-C5' (3C). Thus it can be said from the results of interpretation of Proton and Carbon NMR spectrum obtained atomic H = 14 and C = 16 (Silverstein, Bassler and Morrill, 1981; Oka, 1998).

Mass Spectroscopy obtained molecular ion at m/e = 270 indicates the relative molecular mass of Pinostrobin and fragmentation of m/e = 252 (*-H\(_2\)O), m/e = 242 (M*- CO), m/e = 227 (M + - CO - CH\(_3\)), m/e = 213 (M + - CO - C\(_2\)H\(_5\)), m/e = 193 (loss of ring B/phenyl which reinforced the fragmentation of m/e = 77), m/e = 166 (M* - C\(_2\)H\(_5\) - C\(_6\)H\(_5\)), m/e = 152 (M* - C\(_2\)H\(_5\) - C\(_6\)H\(_5\) - CH\(_3\)) and m/e = 123 (M*-C\(_2\)H\(_5\)-C\(_6\)H\(_5\)-CH\(_3\)-CO), by the following scheme (Silverstein, Bassler and Morrill, 1981, Oka AP, 1998).
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Surgery Fibrosarkoma

Prior to the treatment of one of the infected mice were sacrificed to prove positive fibrosarcoma fibrosarcoma to see whether histopathological tissue fibrosarcoma with HE staining. The results obtained surgically weighs $= 3.4756$ grams and examination with magnification 400x histopathologi seen many chromatin (polikromatin) which prove the damage suffered as a result of fibrosarcoma cells as shown by the following figure 1:

![Histopathology Fibrosarkoma Cells in Mice Results Induction benzopiren](image)

After treatment for 14 days later mice were sacrificed her cancer tissue were taken for subsequent examination as severe cancer who received treatment, making preparations for the examination polilysin p53, COX, VEGF and paraffin blocks. The results also show that the concentration of pinostrobin oral 80 mg / kg could inhibit the growth of fibrosarcoma with a gram weight of 68.62 % and the cancer drug (control +) there is resistance 95.95 % compared to the negative control which is only given orally CMC - Na, this means pinostrobin potentially be developed as a cancer chemotherapeutic agent as shown in the following table 1:

<table>
<thead>
<tr>
<th>Jenis Perlakuan</th>
<th>Berat Fibrosarkoma (gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Without treatment</td>
<td>3.4756</td>
</tr>
<tr>
<td>CMC-Na [ Control (-)]</td>
<td>4.2765</td>
</tr>
<tr>
<td>Pinostrobin 80 mm/kg WW</td>
<td>1.0665</td>
</tr>
<tr>
<td>Cyclophosphamide [Control (+)]</td>
<td>0.1709</td>
</tr>
</tbody>
</table>

Results painting, inspection and reading of p53 and VEGF expression can be seen in the following table 2 and figure 2:
Table 2. The reading results with p53 and VEGF Immunohistochemical Method

<table>
<thead>
<tr>
<th>No</th>
<th>Jenis Sampel</th>
<th>p53</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positif</td>
<td>negatif</td>
</tr>
<tr>
<td>1.</td>
<td>CMC-Na (Control negatif/C-)</td>
<td>287</td>
<td>122</td>
</tr>
<tr>
<td>2.</td>
<td>Pinostrobin 80 mg/kgWW</td>
<td>299</td>
<td>107</td>
</tr>
<tr>
<td>3.</td>
<td>Cancer Drug</td>
<td>265</td>
<td>250</td>
</tr>
</tbody>
</table>

![Histologi VEGF Control (-)](image1)
![Histologi VEGF Pinostrobin 80 mg/kgBB](image2)
![Histologi VEGF Control (+)](image3)

Figure 2. Results Immunohistochemistry of VEGF treatment results

Based on the pictures and the table above shows the results of treatment with VEGF expression Pinostrobin 80 mg / kg reduced the number of blood vessels compared to the number of blood vessels in the negative control (no treatment). This proves that Pinostrobin can inhibit angiogenesis through decrease in the number of blood vessels (Pratiwi D. 2009; Puspita N. 2009).

Conclusion

Based on the above results it can be concluded is:

1. Isolated compounds obtained are pinostrobin or 5-hydroxy-7-methoxy flavanones
2. Pinostrobin potential to be developed as a cancer chemotherapy because of fibrosarcoma weight decreased more than 50 % and in the statistical analysis turns pinostrobin have a significant effect (p < 0.05) in inhibiting cancer growth
3. Histopathology examination fibrosarcoma cells by HE staining in get that there are a lot of chromatin (polikromatin) on fibrosarcoma as evidence of damage to normal cells due to fibrosarcoma
4. Oral pinostrobin concentration 80 mg / kg body weight can reduce 68.62 % by weight of fibrosarcoma and the cancer drug (control +) decreased 95.95 % , and this concentration can increase the expression of p53 so that apoptosis can take place and decreased the expression of VEGF signaling can be inhibited angiogenesis or as antiangiogenesis, this means pinostrobin potentially be developed as a cancer chemotherapeutic agent.
Advice

Need to do more research on apoptosis and antiangiogenesis effects of treatment results above by immunohistochemistry to look at expression of COX-1, COX-2 and MMPs – 9.

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