ANTISEIZURE AND NEUROTOXICITY EFFECTS OF LIGUSTROSID GLYCOSIDE ISOLATED FROM Fraxinus graffithii Clarke COMPARED TO PHENYTOIN AND DIAZEPAM

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ABSTRACT
A study was conducted to determine antiseizure and neurotoxicity effects of Ligustrosid (A CNS Active substance) isolated from Fraxinus Graffithii Clarke compared to Phenytoin and Diazepam (antiseizure standard). Phenytoin has only Anti MES effect. Diazepam has anti MES, anti Metrazol, anti Strychnine, anti Bicuculline, and anti INH effect. Ligustrosid has anti MES,anti Metrazol, anti Strychnine, anti Bicuculline, and anti INH effect. Therefore Ligustrosid has broad spectrum of antiseizure effects than Phenytoin and Diazepam. Clinically, it may be predicted that Ligustrosid will have clinical efficacy against various seizure type. i.e.Generalized Tonic Clonic, Partial seizure, Myoclonic seizure, Absence seizure, and Status epilepticus. Neurotoxicity study proved that Ligustrosid has minimum neurological deficit and other motoric disturbances (TD 50 neurotoxicity = 1413.63 mg/BW). This dosage is much higher than antiseizure activity dosage of Ligustrosid (290-466 mg/kg, BW i.p).Finally it is concluded that Ligustrosid was antiseizure candidate with no neurological deficit effect.

Keywords: Antiseizure, neurotoxicity, ligustrosid, phenytoin, diazepam

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INTRODUCTION
Fraxinus griffithii Clarke (trees: 10 – 20 m) usually grows in forest margins, near villages, by rivers; 100–2000 m in several regions in Bangladesh, India, Ryukyu Islands, Myanmar, Philippines, Vietnam and Indonesia. In Java island, Fraxinus griffithii Clarke is known as Pohon Tiken (Kabupaten Lumajang), Bedali Gombong (Kabupaten Probolinggo), and Pohon Orang Aring (Perkebunan Pancur Angkrek, Prajekan) (Heyne, 1987). Extract of the bark and the leaves of Fraxinus griffithii Clarke (so called: ekstrak Tiken) have been used as an adulterant of illegal opium in certain areas in Indonesia (Boerlage and Kooders, 1987; Heyne,1987; Sutarjadi and Norcholis,1973; Wehmer, 1931). Phytochemical studies found that Tiken extract contains saponin, tannine, and glycosides, and no alkaloids (Sutarjadi and Norcholis, 1973). Survey in 1975 reported that the workers who prepared this extract felt sedation and sometimes went to sleep (Ahaditomo,1975). Explorative study in mice showed that Tiken extract caused little sedation, and reduced motor activity (Ma’riffin, 1975). Furthermore, Basori et al (1998) found that Tiken extract 1500 mg/kg BW up to 2500 mg/kg BW given orally to animals caused little sedation, reduced locomotor activity, did not cause coordination disturbances and muscle paralysis, and did not have hypnogenic effects. All those findings strongly suggested that Fraxinus griffithii Clarke may contain a CNS (Central Nervous System) active substance.

In 1980, Sutarjadi modified the method of Inouye (1975) to isolate Ligustroside glycoside from the bark of Fraxinus griffithii Clarke. For further research and development of Ligustroside to be lead compound of CNS depressant drug, a large amount of Ligustroside and high quality is needed. Basori (1997) modified the method of Sutarjadi to isolate Ligustroside by doing pre extraction process with dichlormethane. This modification proposed to remove all non glycoside compounds from the solution and expected to give a more pure Ligustroside.

The previous pharmacodynamic screening showed that Ligustroside at doses of 100 mg/kg, 200 mg/kg, and 400 mg/kg given intraperitoneally to mice caused little sedation, reduced locomotor activity, did not cause motor coordination disturbances and muscle paralysis, and did not have hypnogenic effect. These results suggested that Ligustroside may have CNS activity as CNS depressant (Basori,1999). But the precise pharmacodynamic profile of its CNS depressant has not been studied in detail yet. Further research is needed to
explore pharmacodynamic profile of its CNS depressant effect.

A very detailed pharmacodynamic study was conducted by Basori (2000), to explore the profile of CNS depressant effects of Ligustrosid. Those studies concluded that Ligustrosid has pharmacodynamic activity as CNS depressant, did not have neuromuscular blocking properties. The primary site of action of its CNS depressant is in the brain, and the mechanism of action may involve the enhancement of GABA-facilitated inhibition in the brain neurons. Pharmacodynamic study with electrical stimulation indicated that Ligustrosid has anti-seizure effect and to prevent seizure spreads on animals (Basori, 2004). Further study, proved that Ligustrosid has antiseizure effects against chemically induced seizure. Pharmacologically, Ligustrosid has been proved to have antiseizure effects. (Basori, 2008). The present research aims to determine antiseizure and neurotoxicity spectrum of Ligustrosid compared to Phenytoin and Diazepam (Antiseizure standard).

MATERIALS AND METHODS

Material used for the present work was dried bark of Fraxinus graffitti Clarke (Pohon Orang Aring). Wet bark was collected from plants used as shade trees at a Pancur Angkrek coffee plantation of PTP XXVI located near Prajekan, East Java. The bark was cut into pieces and dried at room temperature, avoiding direct sunlight, for 1 month. This was done in order to keep active substances from degradation by direct sunlight. Later on, the dried bark was milled with a milling machine into smaller particles, sieved into pulves, and stored in tightly sealed plastic bottles. Ligustrosid was isolated, identified and purified according to the previous method (Basori, 1999). Male BULB/c mice (25–30 g, age: 2 months) used in all experiments were purchased from Pusvetma Surabaya. Animals were acclimatized for at least one week before starting the experiments. They were kept in a soundless room with normal room temperature. During the experiments the animals were not allowed to drink or eat. Finally, the animals were given a code for experiments.

The following drugs and dosages were used: Ligustrosid 100, 200, 300, 400 and 500 mg/kg. This drug was dissolved in solutio Petit (10% absolut alcohol:20% Propylen glycol : 80% water pro injection) and administrated to animals by i.p. injection. Control animals (positive and negative control) were dosed with Solutio Petit and water pro injection. The previous study found that solutio Petit did not have antiseizure effects (Basori, 2000). Epilepsy animal model were made by electrically and chemically induced seizure. Electrically induced seizure was made stimulus of 60 Hz alternating current of 50 mA was applied to the animals through corneal electrodes for 0.1 second with Maximal Electroschock Seizure apparatus (M.E.S). Chemically induced seizure were made by intraperitoneal injection of various epileptogen,i.e CD90 (Convulsive Dose) Metrazol (66.8 mg/BW i.p), Bicuculline (3.2 mg/BW i.p), Strychnine Nitrate (0.88 mg/BW i.p), INH (136.3 mg/BW i.p), Pierotoxine (2.62 mg/BW ip) (Steve White et al,1995;McDonald and Meldrum (1995);Brazil and Pedley (1995); Cortez and Carter (2006), Mares and Kubova (2006), Velisek (2006).

For Phenytoin study, animals were randomly divided into 4 main groups. Each groups of consists of 10 animals. Each groups were given Phenytoin 4 mg/BW , 5 mg/BW, 6 mg/BW, 7 mg/BW, and 8 mg/BW i.p. For Diazepam study, animals were randomly divided into 4 groups (each grup consists of 5 sub groups). Every sub groups consists of 10 animals. Each sub groups of animals were given Diazepam 8 mg/kg BW, 10 mg/kg BW, 12 mg/kg BW, 15 mg/kg BW, and 20 mg/kg BW i.p. On each groups, 60 minutes after Phenytoin administration, and 30 minutes after Diazepam administration, a 60 Hz alternating current of 50 mA was applied to the animals through corneal electrodes for 0.1 second with maximal electroshock seizure apparatus.

Electroconductivity was enhanced with two drops of 0.9% NaCl on each eye. Measurements were done against seizure patterns (clonic flexion, tonic hind leg extension, and clonic). The main measurable component of this experimental model is tonic hind-leg extension (THE) (Kroll,1978; Swinyard,1972; Swinyard et al,1985; Leppik,1993,1994; White,HS et al,1995). Abolition of tonic hindleg extension after drug treatment was regarded as the end point of protection. The calculation of ED50 anti MES (a dose that protects 50% of the animals against electroshock - induced tonic hindleg extension), and the statistical analyses were performed according to Probit methods by using SPSS 9.

For Phenytoin study, animals were randomly divided into 3 groups (each group consists of 10 mice). Each groups of animals were given Phenyoitin 10 mg/kg BW, 30 mg/kg BW, and 100 mg/kg BW intraperitoneally. For Diazepam study, animals were randomly divided in to 3 groups (consists of 10 mice). Each group of animals were given Diazepam 0.025 mg/kg BW , 0.05 mg kg/BW, 0.10 mg kg/BW, and 0.20 mg/BW i.p. Sixty minutes after given Phanytoin, and 30 minutes after given Diazepam, all animals were injected with Metrazole 66.75 mg/kg BW i.p (Convulsive Dose 90).
The capability of a substance to protect animals against clonic seizure regarded as anti – Metrazole activity (Anti Met effect) (Krall, 1978; Swinyard, 1972; Swinyard et al, 1985; Leppik, 1993,1994; White, HS et al, 1995). The calculation of ED50 anti Metrazol (a dose that protects 50% of the animals against Metrazol induced clonic seizure), and the statistical analyses were performed according to Probit method by using SPSS 9.

For Phenytoin study, animals were randomly divided into 3 groups (consists of 10 mice). Each group of animals were given Phenytoin 10 mg/kg BW, 30 mg/kg BW, and 100 mg/kg BW i.p. For Diazepam study, animals were randomly divided into 3 groups (consists of 10 mice). Each group of animals were given Diazepam 0.20 mg/kg BW, 0.25 mg/kg BW, 0.30 mg/kg BW, and 0.40 mg/kg BW i.p. Sixty minutes after given Phenytoin, and 30 minutes after given Diazepam, all animals were injected with Bicuculline 3.18 mg/kg BW i.p (Convulsive Dose 90). The capability of a substance to protect animals against clonic seizures regarded as anti – Bicuculline test (Swinyard et al, 1985; Leppik, 1993, 1994; White, HS et al, 1995).

For Phenytoin study, animals were randomly divided into 3 groups (each group consists of 10 mice). Each group of animals were given Phenytoin 10 mg/kg BW, 30 mg/kg BW, 100 mg/kg BW and 0.40 mg/kg BW i.p. For Diazepam study, animals were randomly divided into 3 groups (each group consists of 10 mice). Each group of animals were given Diazepam 0.10 mg/kg BW, 0.20 mg/kg BW, 0.30 mg/kg BW and 0.40 mg/kg BW i.p. Sixty minutes after given drug, and 30 minutes after given Diazepam, all animals were injected with Picrotoxinine 3.20 mg/kg BW i.p (Convulsive Dose 90). The capability of Phenytoin and Diazepam to protect animals against clonic seizures regarded as anti – Picrotoxin test (Swinyard et al, 1985; Leppik, 1993, 1994; White, HS et al, 1995). The calculation of ED50 anti Picrotoxin and the statistical analyses were performed according to Probit method by using SPSS 9.

For Phenytoin study, animals were randomly divided in to 3 groups (consists of 10 mice). Each group of animals were given Phenytoin 10 mg/kg BW, 30 mg/kg BW, and 100 mg/kg BW i.p. For Diazepam study, animals were randomly divided in to 3 groups (consists of 10 mice). Each group of animals were given Diazepam 0.1 mg/kg BW, 0.2 mg/kg BW, 0.3 mg/kg BW, and 0.4 mg/kg BW i.p. Sixty minutes after given Phenytoin, and 30 minutes after given Diazepam, all animals were injected with INH 136.28 mg/kg BW i.p (Convulsive Dose 90). The capability of a substance to protect animals against clonic seizures regarded as anti – INH test (Swinyard et al, 1985; Leppik, 1993, 1994; White, HS et al, 1995).

For Phenytoin study, animals were randomly divided into 3 groups (consists of 10 mice). Each group of animals were given Phenytoin 10 mg/BW, 12 mg/BW, 30 mg/BW and 100 mg/kg BW i.p. For Diazepam study, animals were randomly divided into 3 groups (consists of 10 mice). Each group of animals were given Diazepam 10 mg/BW, 12 mg/BW, 15 mg/BW and 20 mg/kg BW i.p. Sixty minutes after given Phenytoin, and 30 minutes after given Diazepam, all animals were injected with Strychnine Nitrate 0.88 mg/kg BW i.p (Convulsive Dose 90). The capability of a substance to protect animals against maximal tonic extensor regarded as anti – Strychnine Nitrate test (Swinyard et al, 1985; Leppik, 1993, 1994; White, HS et al, 1995).

For Phenytoin study, The animals were randomly divided into 4 groups (each group consists of 10 mice). Each group of animals were given Solutio Petit 0.1ml/10 g BW, Phenytoin 10 mg/BW, 30 mg/BW and 100 mg/kg BW i.p. For Diazepam study, all animals were randomly divided into 4 groups (each group consists of 10 mice). Each group of animals were given Solutio Petit 0.1ml/10 g BW, Diazepam 2 mg/BW, 3 mg/BW and 5 mg/kg BW i.p. Animals from each group were placed on Rotarod Apparatus (Natsume) which rotate at speed 6 rpm. Normal animal can stand on rotarod very long. The presence of neurological deficit (i.e, ataxia, sedation, hyperexcitability) showed by unability of animal to stand on rotarod for 1 minute. Every experimental done for three replication. Observation done 60 minuteafter given drug. The calculation of TD50 nuerotoxicity of Phanytoin and Diazepam were performed according to Probit method by using SPSS 9.

The animals were randomly divided into 4 groups (each group consists of 10 mice). Each group of animals were given Solutio Petit 0.1ml/10 g BW, Ligustrosid 600 mg/BW, 700 mg/BW and 900 mg/kg BW i.p. Animals from each group were placed on Rotarod Apparatus (Natsume) which rotate at speed 6 rpm. Normal animal can stand on rotarod very long. The presence of neurological deficit (i.e, ataxia, sedation, hyperexcitability) showed by unability of animal to stay on rotarod for 1 minute. Every experimental done for three replication. Observation done 30 minute after given drug. The calculation of TD50 nuerotoxicity of Ligustrosid were performed according to Probit method by using SPSS 9.
RESULTS

Table 1. Cellular mechanism of various electrical and chemically induced seizures on mice

<table>
<thead>
<tr>
<th>No</th>
<th>Epileptogen</th>
<th>Type of seizures</th>
<th>Cellular mechanism of seizures</th>
<th>Clinical Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bicuculline</td>
<td>Treshold clonic seizures</td>
<td>GABA-A Antagonist</td>
<td>Myoclonic seizure</td>
</tr>
<tr>
<td>2</td>
<td>Metrazole</td>
<td>Clonic- tonic seizures</td>
<td>GABA-A Antagonist</td>
<td>Absence seizure, Myoclonic</td>
</tr>
<tr>
<td>3</td>
<td>Strychnine Nitrate</td>
<td>Tonic extensor seizures</td>
<td>Glycine receptor Antagonist</td>
<td>Generalized Tonic Clonic</td>
</tr>
<tr>
<td>4</td>
<td>INH</td>
<td>Treshold clonic seizures</td>
<td>GABA-transaminase inhibitor</td>
<td>Myoclonic seizure</td>
</tr>
<tr>
<td>5</td>
<td>Picrotoxin</td>
<td>Treshold clonic seizures</td>
<td>Picrotoxin receptor Antagonist</td>
<td>Myoclonic seizure</td>
</tr>
<tr>
<td>6</td>
<td>Maximal Electric Schock seizure (M.E.S)</td>
<td>Flexion – Tonic - Clonic</td>
<td>Sodium Channel activation</td>
<td>Generalized Tonic Seizure, Partial Seizure</td>
</tr>
</tbody>
</table>

Table 2. ED50 Antiseizure effects of Diazepam, Phenyoit, and Ligustrosid

<table>
<thead>
<tr>
<th>Drug</th>
<th>ED50 : mg/kg BB, ip</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenyoit</td>
<td>5.55</td>
</tr>
<tr>
<td>Diazepam</td>
<td>0.07, 0.22</td>
</tr>
<tr>
<td>Ligustrosid</td>
<td>290.88*</td>
</tr>
</tbody>
</table>

Table 3. TD 50 Neurotoxicity of Diazepam, Phenyoit, and Ligustrosid

<table>
<thead>
<tr>
<th>Drug</th>
<th>TD50 Neurotoxicity (mg/Kg/BW,i.p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenyoit</td>
<td>36.28</td>
</tr>
<tr>
<td>Diazepam</td>
<td>3.12</td>
</tr>
<tr>
<td>Ligustrosid</td>
<td>1413.63</td>
</tr>
</tbody>
</table>

DISCUSSIONS

The latest research in epileptology proved that there are correlation amongst clinical efficacy of antiseizure drugs, patophysiology of seizure discharges, and antiseizure mechanism. Antiseizure drug which has anti Metrazol effect, clinically are very effective against Generalized Tonic Clonic, Partial seizure, Absence seizure, Myoclonic seizure, and Status epileptics. While anti seizure drug which has anti MES effect, clinically are very effective against Generalized Tonic Clonic, Partial seizure, and Status epilepticus. (Rogawski and Porte,1991; Porte,1993, Cortez and Carter, 2006, Mares and Kubova,2006, Velisek,2006)

In order to study antiseizure spectrum of Ligustrosid, we studied of antiseizure effects and neurotoxicity profile of Ligustrosid compared to Phenyoit and Diazepam as anti epileptic standard. Phenyoit is antiseizure drug which has anti MES effect. While Diazepam is anti seizure drug which has effects anti Metrazole, Anti INH, anti Bicuculline, Anti Strychnine,anti MES. From the previous results (Basori, 2000,2004,2008), it was proved that Ligustrosid has anti MES, Anti Metrazol, Anti Bicuculline, Anti INH, and Anti Strychnine test (Table 1). These results strongly proved that Ligustrosid has broad spectrum of antiseizure effects than Phenyoit and Diazepam. If these results were correlated with clinical efficacy, it may be predicted that Ligustrosid will has clinical efficacy against various seizure type. i.e, Generalized Tonic Clonic, partial seizure, Myoclonic seizure, Absence seizure, and Status epilepticus. In this case, the antiseizure profile of Ligustrosid is nearly similar with...
profile of the third generation anti seizure drugs, which have anti MES, anti Met, anti Bicuculline, anti Picrotoxine, anti Strychnine, and anti INH effects (Cortez and Carter, 2006; Mares and Kubova, 2006; Velisek, 2006).

Neurotoxicity study with Rotarod (Natsume), principally to know the effects of Ligustrosid on neurological deficit, and other motoric disturbances. In this research, proved that Ligustrosid showed unable to stay any longer on rotating rod of instrument at dosage 856.10 mg/kg BW, i.p. This dosage is larger than anti seizure dosage of Ligustrosid (290 - 466 mg/kg,BW i.p). According to probit analysis, TD50 neurotoxicity of Ligustrosid is 1413.63 mg/kg BW, i.p (Table 2). This meant that minimum neurological deficit of Ligustrosid is below the dosage of anti seizure activity. Finally it is concluded that Ligustrosid is antiseizure candidate with no neurological deficit.

CONCLUSION

Comparative study proved that antiseizure spectrum of Ligustrosid is more broadly than Phenytin and Diazepam. More over, neurotoxicity study showed that Ligustrosid has minimal neurological deficit and other motoric disturbances compared to Phenytin and Diazepam. Clinically, it is strongly concluded that Ligustrosid would be very effective against generalized tonic seizure, partial seizure, absence seizures and myoclonic seizures. Further research in pre clinical stage will be needed to investigate the whole pre clinical pharmacological profile of Ligustrosid.

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