

1 RESEARCH ARTICLE

2 Effects of *Pongamia pinnata* on Lipid Peroxidation
3 Products and Antioxidants in Hyperammonemic
4 Rats: with Reference to Circadian Variations

5 MOHAMED MUSTHAFA MOHAMED ESSA and PERUMAL SUBRAMANIAN

6 For author affiliations, see end of text.

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9 ABSTRACT

10 Effects of *Pongamia pinnata*, an indigenous plant used in Ayurvedic Medicine in India on the temporal
11 variations of circulatory lipid peroxidation products and antioxidants in ammonium chloride-(AC)-induced
12 hyperammonemic rats has been studied. Experimental rats were divided into control, AC-treated, those
13 treated with AC + ethanolic leaf extract of *P. pinnata* (PPEt), and PPEt-treated. The characteristics of 24 h
14 rhythms (acrophase, amplitude and mesor) of lipid peroxidation products (thiobarbituric acid reactive sub-
15 stances – TBARS) and antioxidants (reduced glutathione (GSH), glutathione peroxidase (GPx), superox-
16 ide dismutase (SOD) and catalase (CAT)) were analyzed. Elevated lipid peroxidation (increased mesor of
17 TBARS) associated with decreased activities of antioxidants (decreased mesor of GPx, GSH, SOD and
18 CAT) were found in hyperammonemic rats. Differences were also found in amplitude and 'r' values be-
19 tween the hyperammonemic rats and other experimental groups. These alterations clearly indicate that
20 temporal redox status could be modulated by PPEt during hyperammonemic conditions, which may also
21 play a crucial role in disease development.

22 **Keywords:** Hyperammonemia, *Pongamia pinnata*, Circadian, Lipid peroxidation, Antioxidants

23 *Pongamia pinnata* (Linn) Pierre (Leguminosae), 46 furanodiketones and flavonoid glucosides [7-10]. It is a
24 popularly known as 'Pongam' in Tamil and 'Karanja' in 47 well-documented fact that most medicinal plants are
25 Hindi, is a medium sized glabrous tree, found through- 48 enriched with phenolic compounds and bioflavonoids
26 out India and further distributed eastwards, mainly in 49 that have excellent antioxidant property.
27 the littoral regions of South Eastern Asia and Australia 50 Hyperammonemia is a heterogenous group of disor-
28 [1]. In the Indian Ayurvedic literature, various parts of 51 ders characterized by elevated levels of ammonia caus-
29 this plant have been recommended as a remedy for vari- 52 ing irritability, somnolence, vomiting, seizures, de-
30 ous ailments. Different parts of the plant have been used 53 rangement of cerebral function, coma and death [11-13].
31 in traditional medicines for bronchitis, whooping cough, 54 It is a major contributing factor to neurological abnor-
32 and rheumatic joints and to quench dipsia in diabetes 55 malities observed in hepatic encephalopathy and in con-
33 [2]. The leaves are digestive, laxative, anthelmintic and 56 genital defects of ammonia detoxication [11]. Ammonia
34 cure piles, wounds and other inflammations [2]. A hot 57 toxicity results in free radical generation that leads to
35 infusion of leaves is used as a medicated bath for reliev- 58 oxidative stress and tissue damage [14-16] and in-
36 ing rheumatic pains and for cleaning ulcers in gonorrhea 59 creased entry of ammonia to the brain is a primary cause
37 and scrofulous enlargement [3]. Different extracts of 60 of neurological disorders associated with hyperam-
38 leaves, roots and seeds (ethanol, petroleum ether, ben- 61 monemia, such as hepatic encephalopathies, Reye syn-
39 zene extracts and others) of *Pongamia pinnata* have 62 drome, several other metabolic disorders, and some
40 been reported to have anti-inflammatory activity and 63 toxic encephalopathies [14,16]. Oxidative stress is
41 also used to treat infectious diseases such as leu- 64 evolving concept in ammonia neurotoxicity, and the
42 coderma, leprosy, lumbago, muscular and articular 65 potential involvement of oxidative as well as nitrosative
43 rheumatism [4-6]. In addition, phytochemical examina- 66 stress in the deleterious effects of ammonia on the cen-
44 tions of this plant indicated the presence of furanofla- 67 tral nervous system has been recently reviewed [17].
45 vones, furanoflavonols, chromenoflavones, flavones, 68 Oxidative stress mediated lipid peroxidation is one of

Table 1. Changes in the temporal characteristics of TBARS in control and experimental rats

Groups	Acrophase (h)	Amplitude (nmol/ml)	Mesor (nmol/ml)	'r' value	'p' value
Normal	20:3	0.2	2.7	0.69 ^{dr}	<0.001
AC	16:7	0.3	4.5	0.21 ^{ns}	<0.5
AC+PPEt	18:59	0.5	3.0	0.49 ^{dr}	<0.05
PPEt	20:50	0.2	2.8	0.62 ^{dr}	<0.001

dr – detectable rhythmicity; ns- nonsignificant.

Table 3. Changes in the temporal characteristics of SOD in control and experimental rats

Groups	Acrophase (h)	Amplitude (U ^A /mg Hb)	Mesor (U ^A /mg Hb)	'r' value	'p' value
Normal	12:00	0.4	2.9	0.79 ^{dr}	<0.001
AC	13:29	0.2	1.8	0.24 ^{ns}	<0.5
AC+PPEt	12:35	0.1	2.5	0.61 ^{dr}	<0.05
PPEt	11:33	0.4	2.8	0.86 ^{dr}	<0.001

dr – detectable rhythmicity; ns- nonsignificant

A- Amount of enzyme required to inhibit 50% of NBT reduction.

69 the key characteristic features of hyperammonemia 104 ited in the Botany Department of Annamalai University.
70 [15,18]. Recent reports have demonstrated enhanced 105 The shade-dried and powdered leaves of *Pongamia pin-*
71 free radical production in cultured astrocytes exposed to 106 *nata* were subjected to extraction with 70% ethanol un-
72 pathophysiological concentrations of ammonia [13], and 107 der reflux for 8 h and concentrated to a semi-solid mass
73 increased superoxide production and reduced activities 108 under reduced pressure (Rotavapor apparatus, Buchi
74 of various antioxidant enzymes shown in the animal 109 Labor Technik AG, Switzerland). The yield was about
75 models of acute ammonia toxicity [14]. 110 24% (w/w) of the starting crude material. In the pre-

76 The concepts of chronobiology have been studied in 111 liminary phytochemical screening, the ethanolic extract
77 various diseases in an attempt to improve the therapeu- 112 of PPEt gave positive tests for sterols, tannins, flavones
78 tic index of drugs [19]. Circadian rhythms of a number 113 and glycosides [21]. The residual extract was dissolved
79 of biological variables influence drug efficacy of dis- 114 in sterile water and used in the investigation.

80 ease treatments. Furthermore, a lack of synchronization, 115 **Animals**
81 or an alteration of circadian clock function, make

82 rhythm peaks and troughs unpredictable and may re- 116 Male albino Wistar rats weighing 180-200 were used
83 quire specific measures for chronotherapy to improve 117 for the study. They were housed in polycarbonate cages
84 therapeutic index. Hence, assessment of the relevance of 118 under standard conditions (22 ± 2°C, humidity of 45-
85 a normal circadian system for a favourable outcome of 119 64%, 12 h light/dark cycles). They were given standard
86 chronotherapy is desirable [20]. Failure to recognize the 120 pellet diet (Hindustan Lever Ltd., Mumbai, India) and
87 biochemical temporal organization may introduce un- 121 water *ad libitum*. All animal experiments were ap-
88 necessary heterogeneity and hinder the full understand- 122 proved by the ethical committee (Vide. No. 273/2004),
89 ing of biological processes during diseased conditions. 123 Annamalai University, India and were in accordance
90 It is in this context, in the current study, that the varia- 124 with the guidelines of the National Institute of Nutrition
91 tions in the temporal characteristics of lipid peroxida- 125 (NIN), Indian Council of Medical Research (ICMR),
92 tion products: thiobarbituric acid reactive substances – 126 Hyderabad, India. Ammonium chloride was purchased
93 TBARS and antioxidants such as reduced glutathione 127 from Sisco Research Laboratories, Mumbai, India. All
94 (GSH), superoxide dismutase (SOD), catalase (CAT), 128 other chemicals used in the study were of analytical
95 and glutathione peroxidase (GPx), in control, hyperam- 129 grade.

96 monemic rats, and PPEt treated rats were analyzed.

130 Experimental Design

97 MATERIALS AND METHODS

98 Plant Material and Extraction

99 The mature green leaves of *Pongamia pinnata* were 134 secutive weeks [22]. Rats were divided into four groups,
100 collected from Chidambaram, Cuddalore District, Tamil 135 eight animals each. Group 1: Control rats. Group 2: Rats
101 Nadu, India. The plant was identified and authenticated 136 intraperitoneally treated with AC (100 mg/kg body
102 at the Herbarium of Botany Directorate in Annamalai 137 weight) [22]. Group 3: Rats treated with AC (100
103 University. A voucher specimen (No.3670) was depos- 138 mg/kg; intraperitoneally) + PPEt (300 mg/kg; orally)

Table 2. Changes in the temporal characteristics of GSH in control and experimental rats

Groups	Acrophase (h)	Amplitude (mg/dl)	Mesor (mg/dl)	'r' value	'p' value
Normal	6:1	2.8	26.7	0.66 ^{dr}	<0.001
AC	9:2	1.4	15.9	0.19 ^{ns}	<0.5
AC+PPEt	7:38	3.5	22.5	0.49 ^{dr}	<0.05
PPEt	5:25	3.1	25.6	0.62 ^{dr}	<0.001

dr – detectable rhythmicity; ns- nonsignificant.

Table 4. Changes in the temporal characteristics of GPx in control and experimental rats

Groups	Acrophase (h)	Amplitude (U ^A /mg Hb)	Mesor (U ^A /mg Hb)	'r' value	'p' value
Normal	8:00	1.4	23.4	0.69 ^{dr}	<0.001
AC	11:1	0.6	12.5	0.21 ^{ns}	<0.5
AC+PPEt	9:33	1.2	20.8	0.58 ^{dr}	<0.05
PPEt	7:39	1.5	24.6	0.64 ^{dr}	<0.001

dr – detectable rhythmicity; ns- nonsignificant.

A- micromoles of GSH utilized/g Hb.

Table 5. Changes in the temporal characteristics of CAT in control and experimental rats.

Groups	Acrophase (h)	Amplitude (U ^A /mg Hb)	Mesor (U ^A /mg Hb)	'r' value	'p' value
Normal	10:10	1.1	2.1	0.70 ^{dr}	<0.001
AC	13:59	0.7	1.6	0.28 ^{ns}	<0.5
AC+PPEt	11:2	1.0	1.9	0.74 ^{dr}	<0.05
PPEt	8:50	1.2	2.0	0.78 ^{dr}	<0.001

dr – detectable rhythmicity; ns- nonsignificant

A- μmoles of H₂O₂ consumed/min/mg/Hb

139[6,22]. Group 4: Rats orally administered with PPEt
140(300 mg/kg).

141 Biochemical rhythms

142 Biochemical parameters such as plasma TBARS
143[23] and hemolysate GSH [24], GPx [25], SOD [26] and
144CAT [27] were performed after the eight weeks of ex-
145perimental study. Blood samples were collected after
146every 4 h from each group of experimental and control
147rats (00:00, 04:00, 08:00, 12:00, 16:00, 20:00 and 24:00
148h) throughout the 24 h period continuously. Minimal
149amount of blood was collected from orbital sinus with
150great care using heparinized tubes. The values (mean ±
151SD) obtained from each group were plotted versus the
152time of blood collection. The characteristics of circadian
153rhythms (acrophase, amplitude and mesor) were ana-
154lysed by cosinor analysis. Acrophase was expressed in h
155and mesor and amplitude were expressed in the same
156units of documented variables.

157

RESULTS

158 The biochemical variables chosen for this study in
159all the groups showed marked fluctuations over 24 h
160period. The characteristics of rhythms, r and p values
161indicating detectable rhythmicity or non-significant
162temporal variations over a 24 h period of all the groups
163are mentioned in Tables 1-5. The circadian patterns of
164TBARS revealed detectable rhythmicity in control and
165group 3 and 4 (Table 1). Detectable rhythmicity was
166found to be insignificant (p<0.5) in group 2 rats. Ele-
167vated mesor values and advanced acrophase was found
168in AC treated rats. Delayed acrophase and decreased
169mesor values are found in groups 3 and 4 when com-
170pared with controls. Amplitude values in groups 2 and 3
171showed increases and decreases in (group 4) when com-
172pared to group 1.

173 The temporal patterns of GSH showed a detectable
174rhythm in control and groups 3 and 4 (Table 2). Detect-
175able rhythmicity was disturbed in AC treated rats.
176Acrophase was delayed in group 2 and advanced in
177groups 3 and 4 when compared to control rats. Mesor
178values were decreased in group 2 and increased in group
1793 and 4 (Table 2). The detectable circadian rhythm of
180SOD was found to be insignificant (p<0.5) in AC
181treated rats (Table 3). Acrophase was delayed in group
182and advanced in group 3 and 4. Mesor values were de-
183creased in group 2 and increased in groups 3 and 4 (Ta-

184ble 3). The detectable rhythmicities of GPx and CAT
185were found to be insignificant (p<0.5) in AC treated rats
186(Table 4 and 5). Acrophase values were delayed in
187group 2 and advanced in group 3 rats. Mesor values
188were decreased in group 2 and increased in groups 3 and
1894.

190

DISCUSSION

191 The biochemical variables chosen in the study ex-
192hibit marked fluctuations over the 24 h period and the
193results of the present study indicated that control and
194experimental group (AC and PPEt treated) rats differ in
195the temporal characteristics. Alterations in period, am-
196plitude, mesor and acrophase were detected in DNA
197synthesis of spleen, liver and bone marrow of diseased
198mice [28]. Our results also revealed that the rhythms in
199animals are not synchronized / exhibited a phasing with
200that of normal rats. This lack of synchronization re-
201flected as an alteration of circadian clock function in
202hyperammonemic rats and may require specific meas-
203ures for chronotherapy to improve therapeutic index.

204 Oxidative stress and related lipid peroxidation; asso-
205ciated membrane damages are the key features of AC
206induced toxicity [14,18]. Enhanced lipid peroxidation
207indicated as TBARS in AC treated rats might result in
208increased mesor values. Circadian or exogenous daily
209variations in metabolism including those related to lo-
210comotor and brain activities, should result in corre-
211sponding temporal patterns of oxidant formation [29].
212Oxidative and antioxidative indices show circadian
213variations [30]. Desynchronised rhythms of oxidative
214and antioxidative indices reported in diseased conditions
215such as glomerulonephritis [31] and diabetes [32]. The

216temporal patterns of TBARS depend on the nature of 24
217h rhythms of (I) lipid peroxidation levels and (II) SOD
218and CAT activities in blood [33,34]. The acrophase al-
219terations observed in group 2 to 4 indicate that exoge-
220nous perturbations could influence the temporal organi-
221zation of TBARS levels by influencing one or more
222factors mentioned above. Decrease in mesor value
223found in group 3 indicate that PPEt could decrease the
224deleterious effects of AC by reducing the formation of
225TBARS during hyperammonemic conditions [22,35-36]
226and also reported that PPEt to inhibit lipid peroxidation
227and scavenge reactive oxygen species [35-40].

228 The increased mesor values of antioxidants in PPEt
229administered to hyperammonemic rats showed that PPEt
230could elevate the levels enzymatic and non-enzymatic
231antioxidants (35-40). The antioxidant such as GSH and
232CAT also exhibit circadian rhythms and showed peaks
233at 08:00 h in experimental animals [30,33-34] and the
234circadian rhythms of SOD and CAT were also previ-
235ously reported in liver and blood [41]. Circadian fluc-
236tuations in plasma [34] and tissue GSH concentrations
237including the liver, brain [42], heart, stomach [30], kid-
238ney [29], gut [43] etc., were reported. Circadian changes
239in glutathione concentration are related to rhythmic sen-
240sitivity towards radical inducers such as, toluene and
241dioxane [44], carbon tetrachloride [45], cisplatin,

- 242 chloroform and acetaminophen etc., were also re-30412.
 243 ported. 305
 244 The diminished GSH levels and decreased activities 306
 245 of SOD, CAT and GPx in hyperammonemic rats re-30713.
 246 flected in decreased mesor values could be due to over 308
 247 utilization of these antioxidants to scavenge the prod-309
 248 ucts of lipid peroxidation. The present study also re-31014.
 249 vealed that non-significant rhythm characteristics in 311
 250 hyperammonemic rats could influence the temporal pat-312
 251 terns of lipid peroxidation and other antioxidant levels 31315.
 252 in rats. Alterations in the circadian variation in the mi-314
 253 totic index and oxidant and antioxidant status of various 315
 254 liver diseases in rats were reported [34,46]. Increased 31616.
 255 mesor values of GSH, SOD, CAT and GPx observed in 317
 256 group 3 indicate that PPEt may have the ability to con-318
 257 trol the formation of free radicals by elevating the levels 31917.
 258 of antioxidants during hyperammonemic and other con-320
 259 ditions [35-40] 321
 260 Knowledge of the circadian rhythms in normal and 32218.
 261 in pathological conditions can be used to improve the 323
 262 understanding of pathophysiological process and thera-324
 263 peutic approach to illness. Adapting chemotherapy de-325
 264 liverly to circadian rhythms has indeed achieved mean-32619.
 265 ingful clinical improvements in chemotherapy tolerabil-327
 266 ity and efficacy. The detectable circadian rhythms of 328
 267 lipid peroxidation products and antioxidants and their 32920.
 268 alterations during AC/PPEt treatments, in the present 330
 269 study, needs further investigation for the diagnosis and 33121.
 270 for improve the therapeutic efficacy of hyperammonem-332
 271 ia. 333
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CURRENT AUTHOR ADDRESSES

Mohamed Musthafa Mohamed Essa, Department of Biochemistry, Faculty of Science, Annamalai University, Annamalai Nagar, Tamil Nadu, India.

Perumal Subramanian, Department of Biochemistry, Faculty of Science, Annamalai University, Annamalai Nagar, Tamil Nadu, India. E-mail: annamalai_rhythm@yahoo.co.in (Corresponding author).