Research article

Anti-inflammatory, Antinociceptive, Antipyretic and Gastroprotective Effects of Calligonum comosum in Rats and Mice.

*Abdallah H.M.I.\textsuperscript{1}, Asaad G.F\textsuperscript{1}, Arbid M.S.\textsuperscript{1}, Abdel-Sattar E.A.\textsuperscript{2}

\textsuperscript{1}Department of Pharmacology, National Research Centre, Giza, Egypt.
\textsuperscript{2}Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

Available Online: 1\textsuperscript{st} June 2014

ABSTRACT
Recent ongoing research is directed to find natural products that possess therapeutic effects and devoid of side effects. Inflammation, analgesia, pyrexia and gastric disturbances are associated with several pathological conditions. The present study aimed to investigate the anti-inflammatory, antinociceptive, antipyretic and gastroprotective effects of one promising herb; Calligonum comosum. The methanol extract (100%) of Calligonum comosum was administered in the current study at two dose levels, 250 & 500mg/kg, p.o. The anti-inflammatory activity was tested in carrageenan-induced paw edema model of inflammation in rats. Analgesic profile was ascertained in acetic acid-induced writhing and hot plate models in mice. The antipyretic activity was assessed using yeast-induced hyperthermia in rats. Ethanol-induced gastric ulcer model was used to determine the gastroprotective activity of the extract. The methanolic extract of C.comosum (500mg/kg) inhibited carrageenan-induced edema in rat paw. The extract at both doses (250 & 500mg/kg) also produced analgesic activity in acetic acid-induced abdominal constriction response in mice. In hot-plate test, C.comosum at both doses did not show any significant effect against thermal nociception in mice. Treatment with C.comosum extract showed a dose-dependent reduction in pyrexia in rats and suppressed the ethanol-induced gastric lesions. The present results suggest that C. comosum possessed anti-inflammatory, anti-nociceptive, and antipyretic activities. Besides, the herb showed protective effect against ethanol-induced gastric lesions probably through increasing antioxidant defense.

Key words: Calligonum comosum, anti-inflammatory, antinociceptive, antipyretic, gastric ulcer.

INTRODUCTION
Inflammation, pain, and fever are very common complications in day to day life of human beings. Drugs which are in use presently for the management of pain and inflammatory conditions are either narcotics e.g. opioids or non-narcotics e.g. salicylates and corticosteroids. All of these drugs present well known side and toxic effects (1). Moreover synthetic drugs are very expensive to develop. On the other hand, plants and their products represent a potential source that serve as lead for the development of novel drugs that are cheaper and devoid of toxic effects (2). Some plants are found and proved to possess anti-inflammatory, analgesic and antipyretic property (3, 4). Calligonum comosum is an Egyptian desert plant which belongs to family Polygonaceae. It has been used in folk medicine to treat abdominal ailments and toothache (5). The plant is used also as firewood that gives smokeless fires and for tanning. The aerial parts of C. comosum were shown to possess anti-inflammatory, antiulcer, cytoprotective (6) and hypoglycemic (7) activities in rats. Previous phytochemical screening of C.comosum extract showed that it is rich in polyphenolic compounds especially flavonoids (8). Flavonoids were found to be a potent anti-inflammatory agent that seems to be attributed to their antioxidant activity (9). Flavonoids also possess antinociceptive and antipyretic activities (10, 11). In addition, they were found to protect the gastrointestinal mucosa from lesions produced by various experimental ulcer models. Therefore flavonoids could have a more effective and less toxic therapeutic potential for the treatment of gastrointestinal diseases (12). This study will investigate for the first time the anti-inflammatory, antinociceptive, antipyretic and gastroprotective activities of the methanolic extract of C.comosum on albino rats and mice.

METHODS
Research Centre which gave its consent in accordance approved by an ethics committee of the National and filtered drinking water ad libitum. This study was

The test subjects were provided with standard pellet diet 160g and albino mice, weighing 20–25g, were obtained from the animal house of the National Research Centre. The test subjects were provided with standard pellet diet and filtered drinking water ad libitum. This study was approved by an ethics committee of the National Research Centre which gave its consent in accordance

with the National Regulations on Animal Welfare and Institutional Animal Ethical Committee (IAEC).

Determination of LD50: The LD50 was determined. Five groups each of six rats will receive the plant extract in doses ranging from 1 to 5 g/kg b.wt. The LD50 of the tested extract was calculated according to the following formula:

\[ LD50 = \frac{Dm \cdot \Sigma (z \times d)}{n} \]

Where:

- \( Dm \) = The largest dose that kill all animals.
- \( z \) = Mean of dead animals between 2 successive groups.
- \( d \) = The constant factor between 2 successive doses.
- \( n \) = Number of animals in each group.
- \( \Sigma \) = The sum of (z \times d).

1/20 and 1/10 of the maximum dose (5 gm/kg b.wt.) that will not cause mortalities in rats for the plant extract was chosen to be used for the biological investigation throughout the study. Thus, the extract was diluted with distilled water before applying to animal studies to be given at two dose levels; 250 & 500mg/kg.

Table 1. Effect of 100% methanolic extract of C.comosum aerial parts on acetic acid-induced writhing reflex in mice:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of writhes</th>
<th>% of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.3 ± 1.5</td>
<td>—</td>
</tr>
<tr>
<td>Indomethacin (10mg/kg bwt)</td>
<td>6.3 ± 0.5*</td>
<td>73</td>
</tr>
<tr>
<td>C.comosum extract (250mg/kg bwt)</td>
<td>10.1 ± 0.9*</td>
<td>57</td>
</tr>
<tr>
<td>C.comosum extract (500mg/kg bwt)</td>
<td>11.9 ± 0.5*</td>
<td>49</td>
</tr>
</tbody>
</table>

All groups were injected 10ml/kg of 0.6% (v/v) acetic acid solution, i.p. Each value represents the mean no. of writhes ± SEM (n=8). *Significantly different from control group (saline) at P<0.05. Statistical analysis was carried out using one-way ANOVA test followed by LSD post hoc test.

Table 2. Effect of 100% methanolic extract of C.comosum aerial parts on thermally induced algesia using hot-plate model in mice:

<table>
<thead>
<tr>
<th>Groups</th>
<th>Reaction time, seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zero time</td>
</tr>
<tr>
<td>Control</td>
<td>12.70 ± 0.96</td>
</tr>
<tr>
<td>Tramadol (40mg/kg bwt)</td>
<td>12.31 ± 1.10</td>
</tr>
<tr>
<td>C.comosum extract (250mg/kg bwt)</td>
<td>13.25 ± 0.45</td>
</tr>
<tr>
<td>C.comosum extract (500mg/kg bwt)</td>
<td>12.50 ± 0.74</td>
</tr>
</tbody>
</table>

Each value represents the mean reaction time ± SEM (n=8). * Significantly different from control group (saline) at P<0.05. Statistical analysis was carried out using one-way ANOVA test followed by LSD post hoc test.

Table 3: Effect of 100% methanolic extract of C.comosum aerial parts on oxidative stress biomarkers in rats:

<table>
<thead>
<tr>
<th></th>
<th>Stomach MDA (nmol/g tissue)</th>
<th>GSH (µmol/g tissue)</th>
<th>Liver MDA (nmol/g tissue)</th>
<th>GSH (µmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>50.90 ± 2.75*</td>
<td>3.22 ± 0.08*</td>
<td>159.58 ± 9.89*</td>
<td>6.66 ± 0.14*</td>
</tr>
<tr>
<td>Ethanol</td>
<td>85.60 ± 7.79*</td>
<td>2.60 ± 0.06*</td>
<td>231.01 ± 7.37*</td>
<td>6.08 ± 0.07*</td>
</tr>
<tr>
<td>C.comosum extract (250mg/kg bwt)</td>
<td>65.32 ± 3.19</td>
<td>3.69 ± 0.09*</td>
<td>205.60 ± 12.63*</td>
<td>6.09 ± 0.13*</td>
</tr>
<tr>
<td>C.comosum extract (500mg/kg bwt)</td>
<td>62.42 ± 5.20*</td>
<td>3.64 ± 0.07*</td>
<td>122.20 ± 4.92*</td>
<td>6.52 ± 0.06*</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>74.86 ± 6.06*</td>
<td>3.55 ± 0.08*</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Each value represents mean ± SEM (n=8). * Significantly different from control group (saline) at P<0.05. Statistical analysis was carried out using one-way ANOVA test followed by LSD post hoc test.

Plant material and extract preparation: The aerial parts of C. comosum were collected from Cairo Alexandria road in May 2009. Herbarium specimen of the plant was identified by the staff of the Department of Biology, Faculty of Science, King Abdulaziz University. The plant materials were air-dried and subjected to grinding, then kept in dark air-tight closed containers till extraction step. Samples of 500 g each were extracted with methanol (100%) using Ultraturrax T25 homogenizer (Janke and Kunkel, IKA Laboratories, Staufen, Germany). The solvents were distilled off under reduced pressure and the extracts were lyophilized and were kept at 4°C till biological tests (8).

Animals: Healthy Sprague-Dawely rats, weighing 130-160g and albino mice, weighing 20-25g, were obtained from the animal house of the National Research Centre. The test subjects were provided with standard pellet diet and filtered drinking water ad libitum. This study was approved by an ethics committee of the National Research Centre which gave its consent in accordance with the National Regulations on Animal Welfare and Institutional Animal Ethical Committee (IAEC).
Acute anti-inflammatory test: The acute anti-inflammatory effect of the methanolic extract was evaluated by the carrageenan-induced rat hind paw edema test (13).

Animal grouping: A total of 24 adult male albino rats of Sprague Dawley strain divided to 4 groups each of 6 animals. The first group served as control and received normal saline. The second group was administered indomethacin (10 mg/kg p.o.) as the standard drug. The third & fourth groups received the methanolic extract of C.comosum at the two dose levels; 250 & 500 mg/kg p.o., respectively. One hour after the oral administration of the extract, all the animals were injected 0.1 mL of 1% (v/v) carrageenan solution in saline at the sub planter area of the right hind paw. The paw volume of each rat was measured using planimeter before carrageenan injection and then followed by hourly measurement up to 4 hours post-carrageenan administration. The percent of edema or its inhibition on each group were calculated as follows:

\[
\text{Edema (E)} \% = \frac{V_t - V_o}{V_o} \times 100
\]

\[
\text{Inhibition} \% = \frac{E_c - E_t}{E_c} \times 100
\]

Where: Vo is the volume before carrageenan injection; Vt is the volume at t hour after carrageenan injection; Ec is the edema of control group; Et is the edema of treated group.

Antinociceptive activity

Writhing test: Animal grouping: A total of 24 adult male albino mice divided to 4 groups each of 6 animals. The
first group served as control and received normal saline (10ml/kg p.o.). The second group was administered indomethacin (10 mg/kg p.o.) as the standard drug. The third & fourth groups received oral administration of 250 & 500mg/kg of C.comosum methanolic extract, respectively. Thirty minutes later, all groups were given 10ml/kg i.p. of 0.6% (v/v) acetic acid solution (14). Five minutes after acetic acid induction, the number of writhes like abdominal muscle contraction, stretching of the hind limbs and trunk twisting were counted for 20 min and expressed as writhing numbers.

Hot plate test: The protocol of determination of analgesic activity using hot plate method was published by Woolfe and Mc Donald (15).

Animal grouping: For this test, 24 adult male albino mice were divided to 4 groups. The first group served as control and received normal saline (1ml/kg p.o.). The second group was orally treated with tramadol (40 mg/kg bwt) as standard drug. The third & fourth groups received the methanolic extract of C.comosum at two doses 250 & 500 mg/kg, p.o., respectively. For 3 consecutive days preceding the experiment, mice were adapted on the hot plate by placing them on a plate maintained at room temperature for 15 minutes. Each animal was placed everyday onto 52 + 0.5°C hot plate to perform the test. Latency to exhibit nociceptive responses, such as licking paws or jumping off the hot plate, was determined 30 and 60 minutes after the administration of the test substances or saline (16). Reaction time (seconds) for thermal pain was measured prior to the administration of tested extract and standard drug (tramadol; 0 time). To avoid tissue damage of the mice paws, cutoff time for the response to thermal stimulus was set at 60 seconds.

Anti-pyretic activity: In this experiment, animals were divided into 4 groups, each consisting of 6 male albino rats of Sprague Dawley strain. Body temperature of each animal was measured from the rectum using digital thermometer. The Brewer’s yeast suspension is known to produce fever in all the rats (17). Rectal temperature was recorded before yeast injection. Each animal was then injected intramuscularly with pyrogenic dose of Brewer’s yeast (1 mL/100 g bwt of 44% yeast suspension in saline). The rectal temperature was then measured 18 hours following the yeast injection was considered as the basal line of elevated body temperature, to which the antipyretic effect will be compared. Rats expressed >0.3°C increase in rectal temperature were considered pyretic and selected to complete the experiment. A single oral administration of the two doses of the tested extract or paracetamol (standard drug) (18) or saline (control) was carried out and the rectal temperature was determined after 30, 60, and 120 minutes of intervention.

Ethanol-induced gastric & liver injuries
Animal grouping: 32 rats were fasted overnight and randomly divided into four equal groups (eight rats per group). Group I (control negative group) comprises rats that received 1 ml saline. Group II (control positive) comprises rats that received 1ml oral administration of 99% ethanol solution in saline (19). Groups III, IV and V comprises rats that received oral administration of ranitidine (C.comosum extract (250 & 500mg/kg) 1hr prior ethanol injection (as described for group II).
One hour after ethanol treatment, blood samples were collected from rat retro-orbital venous plexus. Serum samples were obtained by centrifugation at 3000 rpm for 10 min using the cooling centrifuge (Sigma and laborzentrifugen, 2k15, Germany). The obtained serum was used for determination of liver enzymes; AST and ALT. Rats were sacrificed afterwards, stomachs were removed, opened along the greater curvature and examined for lesions. The ulcer index was evaluated according to severity and scored using an arbitrary scale: 0 = no visible ulcers, 1 = Petechial hemorrhage or minute pin-point ulcers, 2 = one or two small ulcers, 3 = more than two ulcers, with few large ulcers, 4 = more than two ulcers, mainly large ulcers. Thereafter, stomachs were collected and liver specimens were dissected out. All tissue samples were homogenized in ice-cold 0.9% w/v saline using a homogenizer (glas-Col homogenizer, Terre Haute, USA) to obtain 20% homogenate. An aliquot of the homogenate was immediately used for estimation of oxidative stress biomarkers. Lipid peroxides & GSH contents were assayed colorimetrically according to the methods of Ohkawa et al. (20) & Ellman (21), respectively.

Statistical methods: Statistical analysis for all tests (except gastric ulcer number & severity) was carried out using one-way analysis of variance followed by LSD post hoc test using SPSS software, version 14.0 (SPSS Inc., Chicago, Illinois, USA) (22). For gastric ulcer number & severity tests, statistical significance was determined by Kruskal-Wallis non-parametric one way analysis of variance (ANOVA) followed by Mann Whitney multiple comparisons test. Data were represented as mean + SEM (standard error of mean) of responses. The P-values less than 0.05 were considered to be significant.

RESULTS
Acute anti-inflammatory test: The edema model was established successfully in the hind paw of rats using 0.1ml of 1%carrageenan. (Figure 1) shows that pretreatment with the C.comosum extract at a dose of 500mg/kg resulted in significant (p <0.05) reduction in paw volume as compared to the control group. C.comosum extract exhibited % inhibition of paw edema by 21%, 24% and 31% after 2, 3 and 4hrs of carrageenan administration respectively. However % inhibition of paw volume was less than that of standard drug, indomethacin.

Antinociceptive activity
Writhing test: Writhing reflex was induced successfully in all rats by acetic acid injection represented by subsequent abdominal constrictions and stretching of hind limbs. As shown in the (table 1), indomethacin (10mg/kg) exhibited inhibiting effect by 73%.

C.comosum extract significantly (p< 0.05) decreased the number of acetic acid-induced abdominal constrictions in a dose dependent manner. It showed writhing inhibition of 57% and 49% at doses of 250 and 500mg/kg.

Hot plate test: (Table 2) shows that tramadol injection (40 mg/kg) significantly increased the retention time in hot plate (18.72 + 0.88 and 20.59 + 0.72) at 30 and 60 minutes, respectively as compared to control group (12.61 + 0.31 and 13.10 + 0.69). However, C.comosum extract did not induce any significant change in the reaction time as compared to the control group.

Anti-pyretic activity: As shown in (figure 2) subcutaneous injection of Brewer’s yeast induced pyrexia in all rats 18h after administration. Administration of the methanolic extract of C.comosum at doses of 250 & 500 mg/kg significantly (p<0.05) decreased rectal temperature (37.5 + 0.14 & 37.2 + 0.11, respectively) as compared to the yeast control group (38.6 + 0.26) after 120 min of induced pyrexia. The antipyretic effect exhibited by the extract at the dose of 500mg/kg was more potent than the effect exerted at a dose of 250 mg/kg bwt. However, the dose of the standard drug paracetamol (150mg/kg) was more potent in its hypothermic effect (36.4 + 0.3) than any dose of C.comosum extract.

Ethanol-induced gastric & liver injuries
Gastric inju: (Figure 3) shows all the rats that received 1 mL 50% ethanol administration had induced formation of significant no. of ulcers as compared to the normal group where no ulcers where found. Pretreatment of the C.comosum extract suppressed the ethanol-induced gastric lesions. Administration of the extract at doses of 250 & 500mg/kg decreased number of ulcers (3.6 + 0.33 & 3.5 + 0.34; respectively vs 8.2 + 0.32 for ethanol control group) and ulcer severity (7.2 + 0.51 & 4.2 + 0.40; respectively vs 21.2 + 0.96 for ethanol control group). The reduction of ulcer number induced by the extract was comparable with that of reference drug, ranitidine (2.7 + 0.17). Ethanol administration also created an oxidative stress status in the stomach tissue. It induced lipid peroxidation which reached 168.2% & 80.7% as compared to the control group. Treatment with C.comosum extract improved oxidative status of stomach tissue when co-administered with ethanol. It decreased lipid peroxides content reached 128.3% & 122.6% at doses 250 & 500mg, respectively as compared to control group. The two doses also increased glutathione content reached 113% & 110.2%, respectively, compared with the control group.

Liver injury: As shown in (table 3), Ethanol injection significantly (p<0.05) increased lipid peroxides content in the liver tissue which reached 144.7% and decreased GSH content which reached 91% as compared with the control group. Administration of C.comosum extract
decreased lipid peroxides content which reached 128.8% & 76.6% at doses 250 & 500mg, respectively as compared to control group. Only the higher dose (500mg/kg) increased GSH content to reach 96.9% compared with the control group.

**DISCUSSION**

The present study reveals that the aerial parts of C. comosum possess significant anti-inflammatory, analgesic and anti-pyretic activities in experimental animals. Inflammation is body’s response to disturbed homeostasis. Inflammatory processes involve the release of several mediators including prostaglandins (PG), histamine, thermo-attractants, cytokines and proteinases (23, 24). Carrageenan-induced paw edema is a well established animal model which has been used to evaluate the effect of non-steroidal antiinflammatory agents, which primarily inhibit the cyclooxygenase involved in prostaglandin synthesis (25). Edema formation due to carrageenan in paw is a biphasic event. The first phase is early mediated by mast cell degranulation and histamine and serotonin release (1-1.5h); the second phase (2 to 5h) is characterized by bradykinin release and overproduction of prostaglandins.

Pretreatment of rats with C.comosum extract (500mg/kg) reduced the paw edema during the late phase of inflammation (figure 1). Hence, it can be inferred that the inhibitory effect of methanolic extract of C.comosum on carrageenan induced inflammation could be due to inhibition of the enzyme cyclooxygenase and subsequent inhibition of prostaglandin synthesis. Previous phytochemical screening of C.comosum extract showed that it is rich in polyphenolic compounds especially flavonoids (8). Flavonoids isolated from some medicinal plants have been proven to possess anti-inflammatory effect (9). It is therefore possible that the anti-inflammatory effect observed with this extract may be attributable to its flavonoid component.

In attempt to assess the antinociceptive effect of C.comosum extract, two different algic models were used; namely acetic acid-induced writhing test and hot plate test. The acetic acid-induced writhing test is generally used to evaluate the peripheral antinociceptive effect of drugs and chemicals (14, 26). In this test, acetic acid causes algia by the releasing endogenous substances including histamine, serotonin, bradykinin, substance P, and prostaglandins, which then stimulate the pain nerve endings leading to the abdominal writhing (27). The hot plate test, however, was employed to test the possible involvement of central nervous system in modulation of pain response by C.comosum extract. Pain is centrally mediated via complex processes including opiate, dopaminergic, descending noradrenergic and serotonergic systems (28, 29). Particularly, thermal nociceptive tests are more sensitive to opioid μ-agonists (30, 31). The current study showed that peripheral antinociceptive effects were achieved by administration of C.comosum extract as indicated by reduction in writhing reflex. However, the extract did not show any activity in the model of central algesia, the hot plate test. Hence, probable involvement of the central nervous system in the analgesic effect of C.comosum extract could be ruled out.

In the antipyretic test, C.comosum extract decreased the elevated body temperature after 2hours of induced pyrexia in a dose-dependent manner. Yeast-induced pyrexia is called pathogenic fever and its etiology is due to production of prostaglandins. The infected or damaged tissue initiates the enhanced formation of pro-inflammatory mediators (cytokines like interleukin 1β, α, β and tumor necrosis factor (TNF)-α) which increase synthesis of PGE2 near pre-optic hypothalamic area thereby triggering the hypothalamus to elevate the body temperature (32, 33). Again, Flavonoids are known to inhibit PG synthesis via inhibition of PG synthetase. Therefore, it appears that the antipyretic action of C.comosum may be ascribed to the flavonoidal polyphenolic portion.

In attempt to test the possible anti-ulcerogenic activity of C.comosum extract, ethanol-induced gastric ulcers rat model was used. Ethanol is well-known as a damaging agent to gastric mucosa in animal and clinical studies. Previous reports offered that ethanol-induced gastric mucosal lesions may be attributed to the possible mechanisms: (1) increase oxygen-derived free radicals (34), (2) direct damage to the mucin layer or mucin synthesis (35), and (3) causing gastric cell’s apoptosis (36). The results of the present study showed that pretreatment with C.comosum extract not only suppressed the ethanol-induced gastric lesions including ulcer number and severity (figure 3) but also inhibited ethanol-induced increased MDA production and GSH depletion in gastric mucosa (Table 3). Our finding here is similar to the reports by Islam et al. (37) and Liu et al. (6). The gastroprotective effect of flavonoidal compounds was mentioned previously (12). Besides their action as gastroprotectives, flavonoids also was found to act in healing of gastric ulcers and additionally these polyphenolic compounds can be new alternatives for suppression or modulation of peptic ulcers associated with H. pylori (38, 39)

In the current study, the effect of ethanol on the liver tissue was also investigated. Ethanol is known to produce liver injury via several mechanisms such as; induction of oxidative stress (40), inflammatory response (41), or promotion of liver scarring (42). Continuous intra-gastric in vivo ethanol feeding protocol in the rat reserves as established model for alcohol-induced liver toxicity. With this model, not only is steatosis observed, which is characteristic of several
animal models, but also inflammation and necrosis occur in 2 to 4 weeks and fibrosis begins to develop in 12 to 16 weeks (43, 44). Although ethanol did not affect liver enzymes in this study, it disrupted antioxidant defense mechanism in the liver tissue. Oral administration of the methanolic extract of C. comosum (500mg/kg) concurrently with ethanol ameliorated the oxidative stress in liver through increasing levels of GSH and decreasing MDA. In consistent with these results, the antioxidant properties of C. comosum were recently reported by Abdel-Sattar et al. (8). This was attributed to its high content of polyphenolic compounds (45).

**CONCLUSION**

It can be suggested that the methanolic extract of C.comosum exhibits anti-inflammatory, antinociceptive, antipyretic and gastroprotective effects. The amelioration of oxidative stress by the extract could be a possible mechanism involved in the protective effect against ethanol induced mucosal as well as hepatic injury. Further studies are in progress to isolate and identify the active compounds that are responsible for these activities.

**DECLARATION OF CONFLICTING INTERESTS**

The author(s) declared no conflicts of interest with respect to the research, authorship, and/or publication of this article.

**REFERENCES**


IJTPR, June 2014– August 2014, 6(2):26-33


