LAWSONIA INERMIS AS NATURAL PRODUCT IN CHEMOPREVENTION OF HEPATOCELLULAR CARCINOMA

Abdel-hamid NM¹, Atef Abd El-Baky*², Mohamed O¹ and Thabet KM¹.

¹Biochemistry Department, Faculty of Pharmacy, Minia University, Minia, Egypt.
²Biochemistry Department, Faculty of Pharmacy, Port Said University, Port Said, Egypt.

ABSTRACT
Somatostatin has a role in the progression and control of neoplastic disease. Hepatoprotective effect and anti-tumor action of Lawsonia inermis is the aim of this study. METHODS: Thirty two hepatocellular carcinoma (HCC) albino mice (20–25 g) were randomly divided into four groups (n=8). The first group doesn't treated and served as control. The second group was received octreotoid (0.1 mg/kg body weight). The third group was received ethanolic extract of Lawsonia inermis (200 mg/dl) mixed with drinking water. The Fourth group was received mixture of octreotoid and ethanolic extract of Lawsonia inermis. The treatment will began at 17th week till the last day of 18th week. All animals were sacrificed after 18 weeks.

RESULTS: there was a significant decrease in mRNA expression of AFP and SSTR2 in treated groups with Lawsonia extract, octreotide or in combination when compared to control group. Also, hepatic content of GSH was significantly increased, while hepatic content of MDA significantly decreased. CONCLUSIONS: Flavonoids of Lawsonia inermis have both antioxidant and cytotoxic effect. The use of both Lawsonia extract and octreotide in treatment of HCC resulted in a good synergistic effect for combined therapy.

KEYWORDS: Hepatoprotective, Lawsonia inermis, Octreotide, Synergistic, Flavonoids.

INTRODUCTION
Liver cancer is the fifth most common cancer in the world and the third most common cause of cancer mortality (Asmaa et al., 2009). Liver cancer may be primary (arising from the liver) or secondary liver cancer (referring to cancer that has spread to the liver, having originated in...
other organs such as the colon, stomach, pancreas, breast, and lung). More specifically, this type of liver cancer is called metastatic liver cancer. Hepatocellular carcinoma (HCC) is a cancer arising from the liver (primary liver cancer). It is also known as hepatoma. The liver is made up of different cell types (for example, bile ducts, blood vessels, and fat-storing cells). However, liver cells (hepatocytes) make up 80% of the liver tissue. Thus, the majority of primary liver cancers (over 90 to 95%) arises from liver cells and is called hepatocellular cancer or carcinoma (Mohammad Hossein Somi., 2005). Hepatocarcinogenesis is a multi-step process involving different genetic alterations leading to malignant transformation of the hepatocyte (Ohata et al., 2003).

AFP is the most established tumour marker in HCC and the gold standard by which other markers for the disease are judged. Under physiological conditions, AFP is a fetal-specific glycoprotein. The serum concentration of AFP declines rapidly after birth and its expression is repressed in adults. Pathologically, patients with chronic liver disease, particularly those associated with a high degree of hepatocyte regeneration, can express AFP in the absence of cancer. Also, AFP is elevated in hepatocarcinogenesis, embryonic carcinomas (Grizzi et al., 2007), in gastric (Chen et al., 2003) and lung cancer (Hiroshima et al., 2002). AFP has been used as a serum marker for HCC (Abelev et al., 1963).

There are three different AFP variants, differing in their sugar chains (AFP-L1, AFP-L2, AFP-L3). AFP-L3, is the main glycoform of AFP in the serum of HCC patients and it can be detected in approximately one third of patients with small HCC (Zhou et al., 2006). Elevated levels of AFP-L3 were associated with a shorter tumor doubling time in comparison with those with low levels of AFP-L3. Moreover, AFP-L3 acts as a marker for clearance of HCC after treatment and as a predictor of recurrence as failure to decline to the normal level indicates residual disease (Yuen et al., 2003). Both total serum AFP and AFP-L3 can be measured simultaneously and estimating the AFP-L3/ AFP ratio is helpful in diagnosis and prognosis of HCC (Wright et al., 2007).

Recently the presence of SSTR 2, 3 and 5 was demonstrated on the surface and in the cytoplasm of hepatic stellate cells isolated from an animal model (Song et al., 2004) the use of long acting octreotide in the treatment of HCC is characterized by safety, mainly for cirrhotic patients with low platelet count and prolonged prothrombine time and reliability, also the use of octreotide has shown to improve survival and the quality of life in SSTR
positive patients with advanced HCC and who have no possibility for other therapeutic modalities such as liver transplantation or surgical resection (Dimitroulopoulos et al., 2007). Somatostatin and its analogs can negatively control cancer growth and spread by interacting with specific tumor cell membrane receptors. Upon activation, somatostatin receptors recruit several membrane adaptors/enzymes and activate/inhibit cytoplasmic targets, which in turn initiate a large variety of signal transduction pathways that drive several antitumor activities. Where it will cause anti-proliferative effects through SSTRs by inhibition of mitogenic signaling of growth factor receptor kinase causing growth retardation due to arrest of cell cycle progression (Min et al., 2008). Downregulation of SSTR transcription may result in loss of a tumor suppressive (Li et al., 2012). Upon somatostatin treatment, dissociation of the sst2-p85 complex results in p85 tyrosine dephosphorylation and PI3 kinase inactivation, and consequent inhibition of cell survival and induction of apoptosis (Bousquet et al., 2006).

Recently a complete long-standing regression of hepatocellular carcinoma has been reported after octreotide followed by lanreotide somatostatin analog treatment. However, somatostatin receptor subtypes expressed in this tumor have not been characterized (Rahmi et al., 2007).

Somatostatin and its synthetic analogues now play an important role in the management of gastrointestinal and pancreatic neuroendocrine tumors via peptide suppression. The overexpression of SSTR in human hepatocellular carcinoma cells has been verified (Reubi et al., 1999). Somatostatin analog improves survival in patients with advanced hepatocellular carcinoma by interacting with its specific receptor 2 and 5 (Li et al., 2012).

Recently the presence of SSTR 2, 3 and 5 was demonstrated on the surface and in the cytoplasm of hepatic stellate cells isolated from an animal model (Song et al., 2004) the use of long acting octreotide in the treatment of HCC is characterized by safety, mainly for cirrhotic patients with low platelet count and prolonged prothrombine time and reliability, also the use of octreotide has shown to improve survival and the quality of life in SSTR positive patients with advanced HCC and, despite the high cost, it seems to be an attractive therapeutic option for those who have no possibility for other therapeutic modalities such as liver transplantation or surgical resection (Dimitroulopoulos et al., 2007).

The development of chemotherapeutic or chemopreventive agents for hepatocellular carcinoma is important in order to help reduce the mortality caused by this disease (Kaufmann and Earnshaw., 2000). Thus, significant research efforts have focused on novel
chemotherapeutic drugs from the plant kingdom in search of cancer inhibitors and cures (Pezzuto, 1997) extracts from natural products such as fruits, vegetables and medicinal herbs have positive effects against cancer compared with chemotherapy or recent hormonal treatments (Wu J et al., 2002).

Genus Lawsonia bears one species, *L. inermis* (*Henna, Mhendi, Shudi, Madurang, Mendi, Manghati, Madayantika and Goranti*) till date, having different synonyms as *alba* and *spinosa* belonging to family Lythraceae (Gupta, 2003). This plant is commonly known as Henna or Mhendi and abundantly available in tropical and subtropical areas. Main chemical components are lawsone, esculetin, fraxetin, isoplumbagin, scopoletin, betulin, betulinic acid, hennadiol, lupeol, lacoumarin, quinone and napthaquinone. Different solvents including methanol, ethanol, acetone, chloroform, hexane and water were used to prepare extracts of henna leaves (Haddad Khodaparast et al., 2007).

Henna leaves, flowers, seeds, stem bark and roots are used in traditional medicine to treat a variety of ailments as rheumatoid arthritis, headache, ulcers, diarrhoea, leprosy fever, anti-inflammatory, diabetes, cardiac disease, hepatoprotective and colouring agent (Ali et al., 1995).

Henna's anticarcinogenic property was reported by using a chloroform extract of Lawsonia inermis by the culture tetrazolium salt (MTT) assay on the human breast, colon and liver carcinogenic cell lines and normal human liver cell lines. The preliminary results showed that henna extract displayed cytotoxic effects against HepG2 (liver cells) and MCF-7 (hormone dependent breast cells). The antioxidative activity of this henna extract was found to be highest compared to vitamin E or [alpha]-tochopherol, attributing to the strong cytotoxic activity of the extract (Endrini 2002).

Present study aims mainly to estimate a hepatoprotective effect and anti-tumor action of *Lawsonia inermis*. Also, effect of its flavonoids on hepatic tissue content of both GSH and MDA in response for treatment, along with histological examination of liver sections in experimental albino mice with HCC.
MATERIALS AND METHODS

2.1. Material
Thirty two albino mice (20–25 g) were selected for the present study. They were obtained from the National Research Centre Cairo, Egypt. They were kept under constant experimental conditions with free access to food and water. They were left for one week for accommodation before starting the study. They were monitored for body weight once a week and the doses were adjusted weekly according to body weight. They were fed with basic diet and allowed to free access of tap water and kept under constant environmental conditions at room temperature. During the period of experiment; animals were kept at 12 h light/12 h dark cycle.

Animals were received sub necrotic single dose of DENA 90mg/kg body weight in 0.9% normal saline I.P. (Amarjit et al., 2007). Animals were randomly divided into four groups (n=8), the first group doesn't treated and served as control. The second group was received octreotoid (0.1 mg/kg body weight) at 17th week till the last day of 18th week (Jia et al., 2003). The third group was received ethanolic extract of Lawsonia inermis (200 mg/dl) mixed with drinking water at 17th week till the last day of 18th week (Dasgupta et al., 2003). The Fourth group was received mixture of octreotoid and ethanolic extract of Lawsonia inermis at 17th week till the last day of 18th week. All animals were sacrificed after 18 weeks.

2.2. Sample Preparation
At the end of time point for each group mice were anaesthetized with diethyl ether and sacrificed. The livers were excised rapidly and divided into three parts. The first part was subjected for RNA preparation, the second part was homogenized in 0.25M sucrose solution and stored at -80°C and the last part of liver was fixed in 10% neutral buffered formalin for histopathological examination.

2.3. Preparation of liver homogenate
Briefly the liver tissues were homogenized in hexane/ propanol (3:2 v/v) and centrifuged. The extract was collected and washed with aqueous sodium sulfate. Supernatant was evaporated and the precipitate was weighed, dissolved in 10 ml hexane and stored at -20°C prior to analysis (Hara and Radin 1978).

Reduced glutathione (GSH) and malondialdehyde (MDA) were determined in liver homogenate. GSH was determined according to Ellman (Ellman 1959) and MDA was determined according to Uchiyama and Mihara (Uchiyama and Mihara 1978).
2.3. RNA extraction: using total RNA kit (Omega bio-tek).

30 mg of liver tissue was homogenized in 400 µl of TRK lysis buffer (20 µl of 2-mercaptoethanol per 1 ml of TRK lysis buffer was added directly before use). Homogenization was done using variable speed homogenizer (Glas-Col, cat No 099CK6424) which is the most preferred method for disrupting and homogenizing tissue samples. The lysate was centrifuged at 13000 rpm for 5 min at room temperature and then mixed with 400 µl of 70% ethanol by vortex. This mixture was applied into the hibind RNA spin column and centrifuged at 10000 rpm for 15 second at room temperature, then 300 µl of RNA wash buffer-I were added and centrifuged. This step were repeated three times and in the last one using 500 µl of RNA wash buffer-II diluted with ethanol. The columns were dried by centrifugation at full speed for 1 min to completely dry the hibind matrix. Finally, the RNA was eluted by adding 100 µl of pre heated at 70°C diethyl pirocarbonate (DEPC)-treated water and centrifugation for 1 min at maximum speed.

2.4. RT-PCR procedure

Using RT/PCR preMix kit (Biorom). The premix tubes have all the components necessary for cDNA synthesis and PCR amplification.

The first strand cDNA was synthesized from mouse liver total RNA by reverse-transcription as following: 1 µg of total RNA and 20 pmol of the reverse primer were mixed in a sterile tube then incubated at 70°C for 5 min and rapidly chilled on ice. The content was transferred into PCR PreMix tube and 20 pmol of the forward primer was added. The volume was completed with DEPC-H2O to 50 µl final volume. The cDNA synthesis reaction was performed by incubating the PreMix tube at 42°C for 60 min, followed by incubation at 94°C for 5 min for inactivation of reverse-transcriptase. The PCR was carried out using the same PreMix tubes that contain the DNA polymerase, amplification was carried out in 28 cycle using Bio metra as follows: Denaturation for 30 Sec at 94°C, annealing for 30 Sec at 55°C and extension for one mint at 72°C, after the initial denaturation at 95°C for 5 min.

2.5. Oligonucleotides used for amplifications

Sequences of mouse AFP and SSTR-2 were obtained from Gene Bank®. These sequences (coding sequence) were used to design the primer pairs and the distance between the two primers was 500 bases. Primers were chosen to contain a GC content of 40-60% and TM 62°C. The primer sets as following.
Somatostatin receptor type 2 primers (SSTR-2) | Alpha feto-protein gene primer (AFP)  
---|---  
Upper primer | Upper primer  
5'-GGG TGT CCT CTC CAT TTG AC-3' | 5'-GAC GGA GAA GAA TGT GCT TAG-3'  
Lower primer | Lower primer  
5'-CCG GCG TAT ATC ATG ATG GG-3' | 5'-CGA CCT TGT CGT ACT GAG CA-3'

2.7. Animal Approval Committee

An approval was taken from the University committee resident in College of Medicine/Minia University. The groups were classified and dosed as mentioned above.

Figure (5-A): The expression of SSTR2 in response to HCC treatment.

The expression of SSTR2 in HCC model treated with ethanolic extract of Lawsonia inermis (200mg/100ml drinking water from day one of DENA injection till the end of the experiment) and/or Octreotide (0.1mg twice daily for the two last weeks of the experiment) in comparison to cancer control group.

*C: Cancer control, animals injected with DENA and not treated
*L: Lawsonia inermis treated group.
*O: Octreotide treated group

Figure (5-B): Percentage change in the RNA expression of SSTR2 in response to HCC treatment.
Percentage change the RNA expression of SSTR2 in response to the treatment with ethanolic extract of Lawsonia inermis and/ or Octreotide.

![Image](image_url)

**Figure (6-A): The expression of AFP in response to HCC treatment.**

The expression of AFP in HCC model treated with ethanolic extract of lawsonia inermis (200mg/100ml drinking water from day one of DENA injection till the end of the experiment) and / or Octreotide (0.1mg s.c daily dose for the two last weaks of the experiment) in comparison to cancer control group.

![Image](image_url)

**Fig(6-B): Percentage change the RNA expression of AFP in response to HCC treatment**

Percentage change the RNA expression of AFP in response to the treatment with ethanolic extract of Lawsonia inermis and/ or Octreotide
Table 2: Effect of treatment with ethanolic Lawsonia extract (200mg/100ml drinking water) and/or Octreotide hormone (0.1mg/kg s.c injection) on hepatic tissue content of reduced glutathione (GSH) and malondialdehyde (MDA).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>GSH (µmol/g tissue)</th>
<th>MDA (µmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cancer control</strong></td>
<td>1.31 ± 0.087</td>
<td>148.6 ± 9.75</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lawsonia extract</td>
<td>2.18 ± 0.099(^{a})</td>
<td>92.85 ± 8.93(^{a})</td>
</tr>
<tr>
<td>Octreotide</td>
<td>1.80 ± 0.13(^{x})</td>
<td>113.6 ± 8.60(^{x})</td>
</tr>
<tr>
<td>Lawsonia extract + Octreotide</td>
<td>2.75 ± 0.10(^{a})</td>
<td>54.08 ± 4.98(^{a})</td>
</tr>
</tbody>
</table>

- Data are presented as means ± SEM, n = 5

*Control*: group of mice injected with DENA only and not treated.

-Multiple comparisons were done using one-way ANOVA followed by Tukey-Karmer as post ANOVA test.

\(a\): Highly significantly different from control group at P < 0.001.

\(X\): Significantly different from control group at P < 0.05

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**Photo (5):** Hepatic tissue section of cancer control animals showing cellular and nuclear pleomorphism, multi nucleated giant cells and increased nuclear/cytoplasm (N/C) ratio (H & E x 100)

**Photo (6):** Hepatic tissue section of Lawsonia treated animals showing marked decrease in the N/C ratio, and less prominent nuclei. (H & E x 100)

**Photo (7):** Hepatic tissue section of Octreotide treated group showing marked decrease in the N/C ratio (H & E x 100)

**Photo (8):** Hepatic tissue section of Lawsonia and Octreotide treated animals showing marked decrease in cellular & nuclear pleomorphism and N/C ratio, and less prominent nuclei (H & E x 100)
DISCUSSION

The clinically applicable SST-analogue octreotide binds with high affinity to the SSTR2 and SSTR5 subtypes only, while these analogues bind with a relatively low affinity to the SSTR3 subtype (Hoyer et al., 1994). Somatostatin and its synthetic analogues, octreotide and lanreotide play an important role in the management of gastroenteropancreatic and neuroendocrine tumors through peptide suppression and antiproliferative in addition apoptosis-inducing mechanisms (De Herder and Lamberts 2002). Octreotide can directly inhibit HCC cell proliferation (Hai et al., 2004).

Due to complications and difficulties of cancer treatment, so many studies were tested the natural substances to prevent or relieve cancer progress (Ozaslan et al., 2009). One of these plants that was the subject of our study was Lawsonia inermis, where it has been reported to have anti-inflammatory effects (Gupta et al., 1986), hepatoprotective actions (Latha et al., 2005) and anticancer activities (Endrni et al., 2007).

Flavonoids containing Lawsonia extract were reported to exhibit antioxidant activity (Ramanathan et al., 1989) and were effective superoxide anion scavengers (Robak and Gryglewski, 1988). Beneficial effect of flavonoids has been described for successful treatment of many health conditions including cancer and liver diseases, where antioxidant were found to protect liver cells against damaging effects of ROS (Ren et al., 2003).

The present study exhibited a decrease in mRNA expression of AFP in treated groups with Lawsonia extract, octreotide or in combination when compared to control group. These results were in a harmony with many studies that reported the suppressive effect of octreotide on AFP resulted from HCC (Gabriel et al., 2007). These results were exhibited a good therapeutic effect of octreotide on HCC and this effect may be due to the antiangiogenic and apoptotic effect of this drug on liver cancer cells (Zou et al., 2009, Ji et al., 2011). Also, this drug promotes apoptosis in human somatotroph tumor cells by activating SSTR2 (Ferrante et al., 2006; Georgiadou et al., 2011), where in basal conditions, phosphorylated SSTR2 directly interacts with P85 regulatory subunit of P13 kinase. Upon somatostain treatment, dissociation of SSTR2-P85 complex results in P85 dephosphorylation and phosphatidylinositol-3 (P13) kinase inactivation and consequent inhibition of cell survival and induction of apoptosis (Bousquet et al., 2006). Somatostatin analog improves survival in patients with advanced hepatocellular carcinoma by interacting with its specific receptor 2 and 5 (Li et al., 2012; Georgiadou et al., 2011). Results also exhibited a good
therapeutic or hepatoprotective effect of *Lawsonia* extract against HCC and this effect may be due to the apoptotic effect of octreotide on liver cancer cells, the apoptotic effect of *Lawsonia* extract is mediated by elevating the level of H$_2$O$_2$ (Sauriasari et al., 2007; Mehmet et al., 2009; Endrini et al., 2011), where, apoptosis was induced by H$_2$O$_2$ increasing ( Kim et al., 2003). While, our results exhibited that the use of both *Lawsonia* extract and octreotide in treatment of HCC resulted in a good synergistic effect for combined therapy. This effect may be due to similarity between them in mechanism of action where both medicatin act through induction of apoptosis.

Apoptotic effect of *Lawsonia* extract and octreotide was supported by histopathological examination, where the hepatic tissues of both groups that treated by *Lawsonia* extract and octreotide showed an increase in number of apoptotic bodies, while the number of apoptotic bodies were highly increased in hepatic tissue section for group that received the combined therapy.

The effect of treatment on mRNA expression of both SSTR2 and AFP  were nearly the same as following: There was a slight decrease in the mRNA expression of SSTR2 in group that received *Lawsonia* extract and group received octreotide, but there was a highly significant decrease in mRNA expression of SSTR2 in group that received mixture of *Lawsonia* extract and octreotide in comparison to the untreated control group. These results were in harmony with the study of Lucia with his colleagues showed that the use of octreotide resulted in mild decrease in SSTR2 mRNA synthesis (Lucia et al., 2007). Somatostatin receptor subtype 2 mRNA expression levels correlate positively with tumor size response to octreotide therapy (Giselle et al., 2008). So, these results suggested that the measurement of SSTR2 can be used as well as AFP to follow up the response of HCC for therapy.

In present study we investigated the change in hepatic tissue content of both GSH and MDA in response for treatment. There was a significant increase in hepatic content of GSH in group that received *Lawsonia* extract and group received octreotide, but there was a highly significant increase in group that received mixture of *Lawsonia* extract and octreotide in comparison to the untreated control group.

Also, our results showed that there was a significant decrease in hepatic content of MDA in group that received *Lawsonia* extract and group received octreotide, but there was a highly significant decrease in hepatic content of MDA in group that received mixture of *Lawsonia* extract and octreotide.
extract and octreotide in comparison to the untreated control group. These results were confirmed with previous studies. Latha with his team reported that the *Lawsonia inermis* extract showed tumor suppressor effect, an increase in hepatic content of GSH and decrease in hepatic content of MDA (Mehmet et al., 2009).

**CONCLUSION**

From our data, *Lawsonia* extract having anticancer activity and this effect can be potentiate octreotide action, where the action of *Lawsonia* may be due to the presence of flavonoids which had been reported to have both antioxidant and cytotoxic effect. So the antioxidants were recommended to be used as adjuvant therapy in treatment of HCC.

**REFERENCES**


