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Synergistic interactions of *Aegle marmelos* leaf, *Emblica officinalis* fruit and *Ocimum sanctum* leaf extracts in the regulation of hyperthyroidism and / or hyperglycaemia

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SUMMARY

The effects of *Aegle marmelos* (Rutaceae) leaf, *Emblica officinalis* (Euphorbiaceae) fruit and *Ocimum sanctum.* (Labiateae) leaf extracts were studied in L-thyroxine (0.5 mg/kg) induced hyperthyroidic mice. Separately combined effects of these three plant extracts and of a commonly used antithyroidic drug, Propyl thiouracil (PTU) were investigated for comparison. Serum concentration of thyroxine (T_4), triiodothyronine (T_3), glucose and the activity of hepatic Glucose 6-Phosphatase (G-6-Pase) were considered as main parameters. Hepatic lipid peroxidation (LPO), superoxide dismutase (SOD) and Catalase (CAT) activities were also studied to reveal the toxic effect of the plant extracts, if any. While exogenous T_4 enhanced serum concentration of T_4, T_3, glucose and the activity of hepatic G-6-Pase, a simultaneous administration of either *A. marmelos* leaf (1.0 mg/kg), *E. officinalis* fruit(30 mg/kg) and *O. sanctum* leaf (50 mg/kg) extracts, to hyperthyroidic animals decreased all these parameters. However, the effects were more pronounced, as nearly normal thyroid function and serum glucose concentration were exhibited when all three plant extracts were administered together. A decrease in LPO and a concomitant increase in SOD and the CAT activities indicated the safe and antiperoxidative nature of the plant extracts, administered either alone or in combination. Our findings reveal that the three test plant materials exhibit synergistic effects without any hepatotoxicity, suggesting their potential use in the amelioration of hyperthyroidism and/ or hyperglycaemia.

Key words: *Aegle marmelos*; *Emblica officinalis*; *Ocimum sanctum*; Synergistic effects; Hyperthyroidism; Hyperglycaemia

INTROUCTION

Thyroid hormones are known to regulate almost all body functions either directly or indirectly (Ganong, 1995). Therefore, deficiency (hypothyroidism) /over secretion (hyperthyroidism) of two thyroid hormones, thyroxine (T_4) & triiodothyronine (T_3) leads to several health problems. Particularly hyperthyroidism, if not treated for a long time, very often ends up with clinical abnormalities including hyperglycaemia, some times referred as thyroid diabetes (Roith *et al.*, 1996).

Although some allopathic medicines including Propyl thiouracil & Neomarkazole are available for the treatment of hyperthyroidism, these drugs normally exhibit side effects, such as myxedema and agranulocytosis. As herbal medicines are considered to be safe as well as effective, in the present investigation an attempt has been made to regulate hyperthyroidism with the use of three plant extracts administered either alone or in combination.

Despite the fact that the synergistic effects of two or more plant extracts are commonly considered to be additive & physiologically more potential than the individual plant extract (Williamson, 2001), reports on the combined effects of plant extracts in

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the regulation of hyperthyroidism are meager (Kar and Panda, 2003; Tahiliani & Kar, 2003b). Therefore in the present investigation effort has been made to reveal the combined effects of three different plant extracts, if any, in the regulation of hyperthyroidism in mice.

Aegle marmelos Linn. (Rutaceae), *Emblica officinalis* Gaertn. (Euphorbiaceae) and *Ocimum sanctum* Linn. (Labiateae) were considered as experimental plants because of the fact that all three plants were earlier found to inhibit thyroid functions in euthyroid laboratory animals (Panda & Kar, 1998; Panda *et al.*, 2002; Kar *et al.*, 2002). However, it was not known, whether they are effective in the regulation of hyperthyroidism. Therefore, the present investigation was made in T_4 induced hyperthyroid animals. Simultaneously effects of the individual plant extracts and of a commonly used antithyroidic drug, Propyl thiouracil (PTU) were also studied for a comparison. Serum concentration of T_4, T_3, glucose and the activity of hepatic glucose 6-Phosphatase (G-6-Pase) were considered as main parameters. Lipid peroxidation (LPO), superoxide dismutase (SOD) and Catalase (CAT) activities were also studied in liver (the primary target organ of a drug) to reveal the toxic effects of the plant extracts, if any.

MATERIALS AND METHODS

Plant materials

Emblica officinalis (Euphorbiaceae) fruits, *Aegle marmelos* (Rutaceae) leaf and *Ocimum sanctum* (Labiateae) leaf were collected locally, identified and the voucher specimens (No. E: 112, A:101 and O:115 respectively) were deposited in the departmental herbarium for future reference.

Preparation of the extracts:

A. marmelos extract (AM) was prepared according to the method of Rao *et al.*, (1995). In brief, fresh leaves were dried under shade and then powdered. Leaf powder (200 g) was mixed with 500 ml distilled water and boiled. After filtration through Whatman filter paper # 40, extract was dried by slow heating and continuous stirring. The yield of the extract was 20%. The leaf extract was dissolved in distilled water (d.w.) and administered orally at a dose of 1 g /kg. (Seema *et al.*, 1996).

E. officinalis (EO) fruits of good quality were air-dried and then ground in to fine powder by pulverization. The dried powder was extracted with 95% ethanol at 60°C by soxhlation as described earlier (Panda *et al.*, 2002). The extract, (approximately 12% of air dried powder) dissolved in d. w. was orally administered at a dose of 30 mg/kg.

Fresh leaves of *O. sanctum* (OS) of dark leaved variety were collected locally and were shade dried. Refluxing the powdered leaves with distilled water performed water extraction and then the extract was vacuum dried. (Panda and Kar, 1998). The yield of this extract was found to be 12% of the air-dried powder. The extracts were dissolved in double distilled water for oral administration and were administered at the dose of 50 mg/kg (Sharma *et al.*, 2002).

Animals

Adult healthy colony breed Swiss male albino mice (30±2 g) were used for experimentation. The animals were maintained under standard condition of housing (27±1°C; 14: 10 Light dark cycle) in polypropylene cages with the provision of mice feed (Golden feeds, New Delhi, India) and water *and libitum*.

Experimental design

Experiment 1. Out of forty male mice, thirty-two were rendered hyperthyroidic by daily subcutaneous injection of L-T_4 at a dose of 0.5 mg/kg./d for 12 consecutive days as used earlier (Panda and Kar, 2000, 2001, 2003). They were then divided into five groups of eight each. While gr. II continued to receive only T_4, gr. III, IV, and V, were treated with equivalent dose of T_4 along with EO (30 mg/kg/d), OS (50 mg/kg/d) and AM (1.0g/kg/d) extracts respectively everyday for fifteen days, while, gr. I animals received only 0.1 ml of vehicle and served as control.

Experiment 2. Thirty-two animals were divided into four groups of 8 each. Twenty four (group II-IV) were rendered hyperthyroidic by daily subcutaneous injection of L-T_4 at a dose of 0.50 mg /kg./d for 12 consecutive days and were then divided in to three groups of eight each. Group II continued to receive only T_4, while gr. III was

treated with equivalent dose of T_4 along with EO (30 mg/kg/d), AM (1.0 g/kg/d) & OS (50 mg/kg) extracts, all together. Group IV was administered (i.p) with Propyl thiouracil (PTU) at a pre-standardized dose of 10 mg/kg/d (Kar *et al.*, 2002) along with T_4. Gr. I animals received only vehicle and served as control (CRL).

All the doses were administered between 10.00 and 11.00 hr of the day to avoid circadian variations and both the experiments were continued for 15 days.

Serum collection and biochemical estimations

Twenty-four hours after the administration of the last dose, animals were sacrificed by cervical dislocation. Blood from each one was collected and serum samples were stored at -80°C until assayed for total T_3 and T_4 by radioimmunoassay (RIA). Liver was removed quickly, washed thoroughly with phosphate buffered saline (PBS, pH 7.4) and processed for biochemical estimations. LPO was estimated by thiobarbituric acid (TBA) reaction with Malondialdehyde (MDA), a product formed due to the peroxidation of membrane lipids, according to the method of (Ohkawa *et al.*, 1979) as modified by Jamall and Smith (1985). LPO was expressed as nM of MDA formed/hr/mg protein. Hepatic SOD activity was assayed according to the method of Marklund and Marklund (1974). The enzyme activity was expressed as units/mg protein and one unit of the enzyme is defined as the activity that inhibits autoxidation of pyrogallol by 50%. Catalase (CAT) activity was estimated following the method of Aebi (1983). For the determination of serum glucose, the modified glucose oxidase method of Hugget and Nixon (1957) was followed using GOD-POD kits from Sigma diagnostic Ltd, Baroda, India. Hepatic G-6-Pase activity was studied by the method of Baginski *et al.* (1974).

Estimation of thyroid hormones and protein:

For the assay of thyroid hormones RIA kits were used, procured from Bhabha Atomic Research Center, Mumbai, India and serum concentrations of total T_3 and T_4 were estimated using routine RIA protocols followed earlier in our laboratory (Panda & Kar, 1999, 2001). In brief RIA was performed using Tris hydroxymethyl amino methane buffer (0.14 M; pH 8.6). The reaction mixture comprised of standard /sample, buffer, ^{125}I-T_4/T_3 antibody . The tubes were incubated at 37°C for 30 min (for T_4)/45 min (for T_3). Centrifugation was at 2000 g for 20 minutes. For radioactivity counting ^{125}I gamma counter was used. Quality controls were also run simultaneously. Lower limits of sensitivity for T_3 and T_4 were 0.07 ng/ml and 0.12 ng/ml respectively. Inter-assay variation was less than 5% for both the hormones.

The hepatic protein content was determined using bovine serum albumin as standard by the method of Lowry *et al.* (1951).

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). For statistical evaluation, analysis of variance (ANOVA) followed by *Student's t-* test was considered. Statistical significance was ascribed only when p-value was less than 0.05.

RESULTS

Experiment-1. Administration of L- T_4 to normal (euthyroid) mice increased the serum concentrations of T_3, T_4, glucose, & the hepatic G-6-Pase activity significantly ($P<0.001$) when compared to the respective control values (Fig. 1).

The oral administration of AM/EO/OS extract in the T_4-induced mice significantly decreased the concentration of serum glucose, T_3 and T_4, ($P<0.001$ for all) with a parallel decrease in hepatic G-6-Pase activity ($P<0.05$, 0.01, 0.001 respectively) as compared to the values of the group treated with T_4 only. While an increase in LPO & decrease in SOD & CAT activities was observed in T_4 treated group ($P<0.001$ for all), reverse observations were made with respect to LPO ($P<0.01$, 0.001 and 0.001 respectively), SOD ($P<0.05$, 0.01 and 0.001 respectively) and CAT ($P<0.001$, for all) activities following the administration of AM/EO/OS extracts along with T_4.

Experiment 2. Here also T_4 administration enhanced the thyroid hormone concentrations, & G-6-Pase activity significantly ($P<0.001$, for all). However, serum T_3, T_4 & glucose concentration were decreased significantly by *Ocimum*+ *Aegle*+ *Emblica* extracts (P, <0.001) as compared to T_4 treated values.

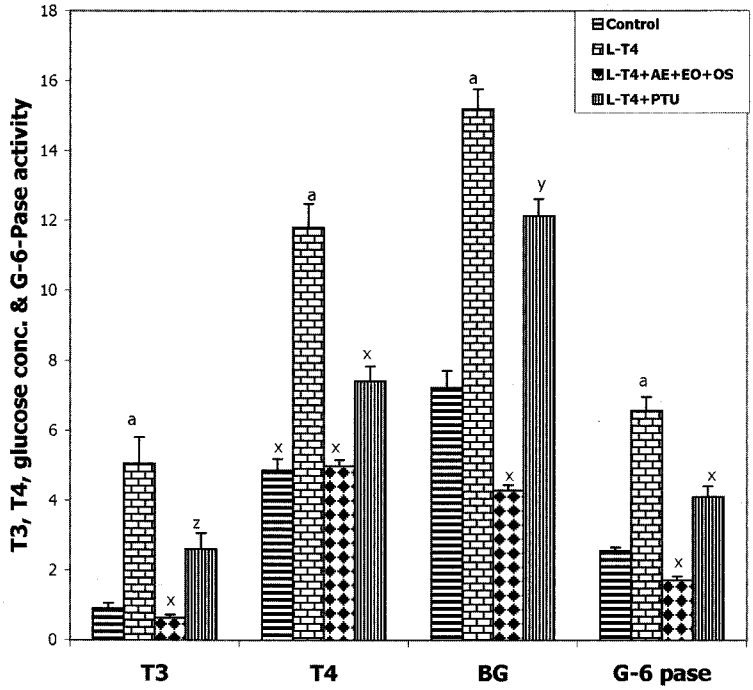

Fig. 1. Changes in serum concentrations of T_3 (ng/ml), T_4 (ng/ml ×10) and glucose (mg%) and hepatic G-6-Pase activity (μM phosphate generated/min/mg protein $\times 10^{-1}$) following the administration of *A. marmelos* (AM)+*E. officinalis* (EO)+*O. sanctum* (OS) extracts or PTU for 15 days in hyperthyroid male mice. Each bar represents the mean ± SEM (n=8). T_4, thyroxine; T_3, Triiodothyronine; G-6-Pase, Glucose 6- phosphatase. $^{a}P<0.001$, $^{b}P<0.01$ and $^{c}P<0.05$ as correspond to their respective control values. $^{x}P<0.001$, $^{y}P<0.01$ and $^{z}P<0.05$ as compared to the respective T_4 treated values.

With respect to LPO, SOD and CAT activities, in these hyperthyroidic animals, receiving three plant extracts, hepatic LPO decreased, while SOD & CAT activities were enhanced significantly ($P<0.001$).

Administration of PTU along with T_4 decreased the concentrations of T_4, T_3, serum glucose and G-6-Pase activity significantly ($P<0.001$, 0.05, 0.01 and 0.001 respectively) as compared to their corresponding values in T_4 treated groups. LPO & SOD activities remained unchanged, whereas, CAT activity was found to be increased significantly ($P<0.01$) in this group. When percent inhibition was calculated out, maximum inhibition in T_3 and in serum glucose concentration was found in the group that received all three plant extracts together (Table 3).

Table 1. Effects of *Aegle marmelos* leaf (AM, 1.00 g/g), *Emblica officinalis* fruit (EO, 30.00 mg/kg) and *Ocimum sanctum* (OS, 50.00 mg/kg) leaf extracts on hepatic LPO (nM MDA formed/h/mg protein), SOD (units/mg protein) and CAT (μM H_2O_2 decomposed/min/mg protein) activities in L-T_4 induced hyperthyroid male mice

Groups	LPO	SOD	CAT
Control	0.87±0.04	6.85±0.40	41.57±1.41
L-T_4	1.81^{a}±0.24	2.38^{a}±0.27	26.84^{a}±0.94
L-T_4+AM	0.91^{y}±0.10	5.05^{z}±0.98	54.76^{x}±3.29
L-T_4+EO	0.47^{x} ±0.03	4.92^{y}±0.67	48.34^{x} ±3.16
L-T_4+OS	0.84^{x}±0.07	6.42^{x}±0.31	43.54^{x}±1.91

Values are mean±SEM. $^{a}P<0.001$ compared with the respective control values; $^{x}P<0.001$; $^{y}P<0.01$; $^{z}P<0.05$ compared with the respective values of T_4 treated group.

DISCUSSION

Results reveal that *A. marmelos, E. officinalis* and *O.* sanctum extracts, when administered individually, reduced serum T_3, T_4 & glucose concentrations as well as hepatic G-6-Pase activity in T_4 induced hyperthyroidic animals, as observed earlier with some other plant extracts (Panda & Kar, 2001, 2003; Tahiliani & Kar, 2003a). Interestingly, combined effects of these plant extracts (results of experiment 2) for most of the parameters studied, were much

Table 2. Combined effects(L-T_4+AM+EO+OS) of *A. marmelos* leaf, *E. officinalis* fruit and *O. sanctum* leaf extracts or Propyl thiouracil (PTU) on hepatic LPO (nM MDA formed/h/mg protein), SOD (units/mg protein) and CAT (μM H_2O_2 decomposed/min/mg protein) activities in L-T_4 induced male hyperthyroid mice

GROUPS	LPO	SOD	CAT
CONTROL	0.96±0.06	5.42 ±0.27	65.86±3.00
L-T_4	1.98[a]±0.19	3.99[a]±0.32	44.04[a]±2.31
L-T_4+AM+EO+OS	0.55[x]±0.13	8.22[x]±0.47	82.09[x]±6.25
T_4+PTU	1.26 ±0.25	5.65 ±0.71	66.23[y]±7.15

Values are mean±SEM. [a]$P<0.001$ compared with the respective control values.
[x]$P<0.001$; [y]$P<0.01$ compared with the respective values of T_4 treated group.

Table 3. Percent (%) decrease on serum of T_3, T_4 and glucose concentrations following the administration of AM, EO, and OS extracts either alone or in combination in relation to the values of L-T_4 treated hyperthyroid male mice

Groups	T_3	T_4	glucose
L-T_4+AM	87.30%	49.05%	35.91%
L-T_4+EO	61.64%	63.87%	27.19%
L-T_4+OS	80.68%	44.14%	59.88%
L-T_4+AM+EO+OS	87.50%	58.00 %	71.80%

more greater than that of individual extract. Although percent reduction in serum T_4 was little more (63%) when *Emblica* extract was administered alone, maximum decrease in T_3 (the metabolically most active thyroid hormone) & in serum glucose concentrations was observed by the administration of all three different plant extracts together. In fact, thyroid function and serum glucose level were nearly normalized in these animals suggesting that these three plant extracts in combination may potentially regulate thyroid diabetes/hyperthyroidism synergistically.

Despite a common belief that the therapeutic efficacy of mixture of herbs is more than the individual plant extract (Williamson, 2001) and the fact that some reports are already available on the effects of some herbal formulations/compounds on other disorders (Mirsalis *et al*, 1993, Ahmed and Sharma, 1997; Pari and Sarravaan, 2002), no investigation was made till to date on the combined effects of three plant extracts in the regulation of thyroxine induced hyperthyroidism /or hyperglycaemia.Therefore the present finding is quite significant to suggest the possible synergistic effects of three different plant extracts in the regulation of hyperthyroidism and /or hyperglycaemia.

When the combined effects were compared with that of a standard antithyroidic drug PTU, the former was found to be more inhibitory to T_3, T_4 & glucose concentrations (87.50. 58.00 & 30.83% respectively) as compared to the latter (48.31, 40.70 & 30.83 % respectively) which further indicate that the said plant extracts in combination may prove to be more effective in regulating hyperthyroidism as compared to the traditional drug, PTU.

It is now well understood that about 90% of the serum T_3 is produced by the extrathyroidal conversion of T_4 in peripheral organs, particularly in liver and kidney (Visser *et al.*, 1978). Therefore, a decrease in T_3 concentrations by individual plant extract or in combination could be due to their efficacy in inhibiting the monodeiodination of T_4, as suggested earlier for some other plant extracts (Panda & Kar, 2001, 2003). On the other hand, as the second thyroid hormone, T_4 is primarily produced in the gland itself, a decrease in serum T_4 concentrations following the administration of the plant extracts could be the result of a direct inhibition of T_4 synthesis & /or release at the glandular level. Another possibility in decrease in T_4 concentration could be an indirect effect through an inhibition in the production of thyroid stimulating hormone and / or thyrotropin releasing factor. Whatever may be the mechanism of action, our findings certainly suggest the potential synergistic effects of the three test plant extracts in regulating thyroid diabetes and thyrotoxicosis.

Hyperthyroidism is commonly associated with the disturbances in carbohydrate metabolism that results in impaired glucose tolerance, increase in peripheral insulin resistance & transient hyperglycaemia (Gorska *et al*, 1989; Ardawi and Khoja, 1993). This condition persists till the excess thyroid hormone is normalized. Interestingly, the three different plant

extracts in combination were able to normalize the thyroid hormones and glucose concentrations in thyroxine induced hyperglycaemic animals, suggesting that the experimental plant materials may have the potency to normalize the thyroid function synergistically.

Lipid peroxidation (LPO), a free radical chain reaction is the most common manifestation of tissue toxicity to various chemicals (Halliwell and Gutteridge, 1989). It is primarily an outcome of oxidations & formation of free radicals by peroxides & superoxides, which are generated continuously in living cells exposed to physiological stress and in this process, LPO alters the normal structural & functional properties of the cell, ultimately leading to cyto-toxicity and dismantling of the membrane structure (Kreps *et al*; 1986). The toxic lipid peroxides accumulated in the system are generally metabolized by cytosolic enzymes such as SOD & CAT to prevent any damage to biological membranes & to maintain cellular integrity (Seiss, 1993). An increase in LPO in response to various disease & tissue damage is maximally observed in liver cells, which are more susceptible to stress induced damage (Emerit & Chaudire, 1989) and the fact that liver is the common target organ of a drug, LPO activity of liver is always necessary for evaluating the possible toxic effect of a new drug. In the present investigation, exogenous thyroxine induced the level of hepatic LPO with a decrease in the activities of SOD &CAT. However, none of the plant extracts were found to increase LPO when administered either alone or in combinations, in T_4 treated animals suggesting that these plant extracts at the doses used, are apparently not toxic.

CONCLUSIONS

Prolonged hyperthyroidism is very often considered to be a cause for hyperglycaemia and diabetes mellitus. Our findings clearly indicate that the synergistic effects of *A. marmelos, E. officinalis* and *O. sanctum* extracts are more pronounced as compared to a standard antithyroid drug, PTU and to the individual extract. As investigated plant extracts were also found to be antiperoxidative in nature, their use for the amelioration of hyperthyroidism and / or hyperglycaemia may be considered. However, further investigation is a must for optimization of the dose and duration for human therapy.

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