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PROTECTIVE EFFECTS OF *GALIUM VERUM* L. EXTRACT ON THE HYPOTHALAMIC – PITUITARY – ADRENAL AXIS UNDER ANAKINETIC STRESS CONDITIONS, IN RATS. HISTOLOGICAL ASPECTS

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ABSTRACT: 24 female Wistar rats (120 ± 10 g) were divided into 4 groups: - control group (C); - *Galium verum* treated group (E), (25 mg extract/100 g bw); - anakinetic stressed group (S); - stress + *Galium verum* extract (25 mg extract/100 g bw) treated group (SE). Results revealed different reactivity of hypothalamo-hypophyseal-adrenal axis in the three experimental groups. The extract administration, in conditions of exposure to stress, resulted in an enhancement of NPV neurosecretory activity – associated with a possible stimulation of CRH release, a possible activation of adenohipophyseal hormones, as well as stimulation of adrenal steroid hormones. In S group, the effects of stress were opposite to those of E and SE groups. Histological results of the study proved that the administration of *Galium verum* vegetal extract in condition of anakinetic stress exposure induced important morphological changes at the all constitutive assembly of hypothalamo-hypophyseal-adrenal axis. These results justify the stimulation of secretory activity of the axis.

Keywords: *Galium verum* extract; rats; hypothalamo-hypophyseal-adrenal axis; anakinetic stress.

INTRODUCTION:

Chronic psychological stress is one of the major factors that contribute to several pathological disorders (Brown, 1993; Cohen *et al.*, 2007; Gouir and Matuszewich, 2005). Immobilization/restraint stress is an easy and convenient method to induce both psychological, an escape reaction and physical stress, a muscle work which leads to restricted mobility and aggression (Lee *et al.*, 2015; Ramanova *et al.*, 1994; Singh *et al.*, 1999).

However, it is well known that intensive stress response results in the production of reactive oxygen species (ROS) e.g. hydrogen peroxide, hydroxyl radical and superoxide anion radical that cause lipid peroxidation, especially in membranes and can play an important role in tissue injury. It has been suggested that chronic stress and high level of glucocorticoids, the adrenal steroids secreted during stress, affect various processes involving ROS and increase ROS by approximately 10% basally (Cohen *et al.*, 2012; Kovacs *et al.*, 1996). Presence and harmful effects of free radicals were imposed to find remedies. Last decades of research have identified substances with antioxidant effects, which may neutralize or limit the harmful effects of free radicals. In this category belong polyphenols, too.

Currently there is an interest for identification of some species of plants with a rich content in polyphenols. The mechanisms of polyphenols action are complex and little known. The most remarkable aspects relate to antioxidant activity which means reducing the free radicals production and lipid peroxidation processes, especially in the cell membranes, which provides protection of the entire cell. Another mechanism of polyphenols action is blocking toxins and free radicals by competitive inhibition for some receptor in the cell membrane.

Medicinal plants and other plants, which contain more polyphenols, are a category of plants sought due to their possible use in phytotherapy. The most popular Galium species of our country with medicinal uses include: *Galium verum* L. and *Galium mollugo* L

Genus Galium, Fam. Rubiaceae, comprises about 300 species spread across the globe. It occupies large areas in Europe and Asia, from the plains to the mountain stage at an altitude of 2,500 m. Romanian flora is among 28 species (Flora RPR, 1961) and 35 species of Galium (Ciocârlan, 2000). These include: 16 species with white flowers, 6 species with yellow flowers, two species with green flowers, two species with purple flowers and two species with undefined flowers. The most known officinal species of spontaneous Romanian flora are the species: *Galium verum* and *Galium mollugo*. Galium species (Lady's Bedstraw or Yellow Bedstraw) are known from the most ancient times as officinal plants with a variety of therapeutic properties: diuretic, depurative, slightly sedative and antispasmodic weak.

Although in Romania have made a series of studies on Galium species (Gîrd and Florea, 2006; Tămaş *et al.*, 2006; Hemcinschi *et al.*, 2008; Hemcinschi *et al.*, 2009; Milića *et al.*, 2013), generally Galium species have not been fully studied in terms of chemical, pharmaceutical (Bruneton, 1993), nor in terms of biological effects which that extracts may have on the animal body.

Due to its chemical compounds: iridioides (asperuloside) with anti-inflammatory, analgesic, sedative effects; flavonosides with diuretic and anti-inflammatory action, phenyl-propane compounds,

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tannins with healing action, citric acid, minerals, enzymes, plant has many therapeutic properties: depurative (blood cleansing), diuretics; cholagogue (improve bile secretion); astringent, healing, anti-inflammatory and vulnerary (healing of wounds); diaphoretic (increases sweat gland secretion); appetizers, flavoring. These therapeutic properties gives the plant a lot of phytotherapeutic uses (Demirezer et al, 2006; Orhan *et al.*, 2012).

Thus, we proposed to study the protective effects of *Galium verum* extract on hypothalamic – hypophyseal - adrenal axis activity in rats under anakinetic stress condition in order to obtain phytopharmaceutical preparations used in phytotherapy to protect this structures which may be potentially affected by stress.

MATERIALS AND METHODS:

Extract preparation

The hydroalcoholic extract of *Galium verum* was obtained at the Faculty of Pharmacy, Cluj-Napoca. The vegetal material (*Galii veri herba*) was harvested on July 2012 from the Becaş Colony meadows, Cluj-Napoca. The vegetal material was dried at room temperature and then crushed to a fine powder (VI FR X sieve). The fluid extract (1 : 1) was obtained with alcohol 70˚ using the percolation technique of Squib (Stoian and Savopol, 1977) using 1 p alcohol for 1 p *Galii verii herba* (pulvis). Qualitative analysis of the polyphenolic compounds (flavonoids, phenyl-propane compounds) of the *Galium verum* extract was done by thin-layer chromatography (TLC), (Wagner *et al.*, 1996). (Table 1.)

Table 1.
Quantitative determination of total polyphenolic, flavonoid, and phenyl - propane compounds from *Galium verum* extract.

Extractive solution	Total polyphenols concentration	Flavonoids concentration	Phenyl - propane compounds concentration
Fluid extract	1040.8 mg gallic acid /100g extract	567 mg rutoside/100 g extract	341.75 mg caffeic acid/100 g extract

Animals

The experiments were performed on mature white Wistar female rats, weighing 120 ± 10 g. Animals were obtained from the „Iuliu Hatieganu" Medicine and Pharmacy University, Cluj-Napoca biobase and kept under standardized zoohigienical conditions, respectively with free access to pet food (standaridsed pellet for rodent, Cantacuzino Institute) and water *ad libitum*, standard environmental conditions and 12 : 12 circadian cycle, in accordance to the European Communities Council Directive 2010/63/UE and according to the approval of the Ethics Committee and Animal Protection for Experiments from the Institute of Biological Research, NIRDBS branch, Cluj-Napoca, Romania.

Experimental design

Animals were divided into 4 experimental groups of 6 animals each, as follows: 1 - control group, C; 2 - extract group (E), treated for 15 days with a hydroalcoholic extract of *Galium verum* in amount of 25 mg extract/100 g body weight (bw), by intragastric gavage, *á jeun*; 3 - anakinetic stressed group, S. The immobilization stress was induced in rats by putting them in 20 cm × 7 cm plastic tubes for 3h/day for 15 days (Marcilhac *et al.*, 1999; Yokus *et al.*, 2005). There are several 3 mm holes at the far end of the tubes for breathing, which allows ample air but animals will be unable to move. Moreover, animals were kept in dark condition in the period of immobilization to emphasize the stress state; 4 - anakinetic stress + *Galium verum* fluid extract treated group (SE). This group was stressed and receved extract of *Galium verum* in same conditions like previous groups. In the 16th day, animals were killed by decapitation after a pre-anesthesia with ether. The brain, pituitary and adrenal glands were removed and then processed according to analyzed morphological or functional parameters.

Histopathological investigations

The brain, pituitary and adrenals were removed and weighed. Fragments of tissues were fixed in Bouin fluid for 24 hours, the parts being processed for inclusion in paraffin (Mureşan *et al.*, 1974). The fragment was sectioned at the Reichert - Austria type microtome with a thickness of 7 µm. The sections were deparaffinized using two changes of 50 ml of xylene for ten minutes at room temperature. The sections were rehydrated in a sequence of 50 ml of 100%, 96%, 70% ethanol, for ten minutes. The tissues were washed once in distilled water, and stained with specific dyes. The general morphology of the brain and to highlight the neurosecretory activity of periventricular regions of the hypothalamus (Nissl granulations), was determined using Kluver-Barrera method (Kluver-Barrera, 1953). The staining was performed using 5% Luxol fast blue 1% cresyl violet (Mureșan *et al.*, 1974), which stains both the nucleoli and the rough endoplasmic reticulum in (neurosecretion) in perikaryons. The histological examination of the slides was performed on an Olympus BX-51 microscope, and the images were captured with a coolsnap-Pro CF color CCD camera (Roper Scientific Photometrics, Tucson, USA) using the Image-proplus 4.1 - 4.5 data acquisition software (Media Cybernetics Inc., Bethesda, USA).

RESULTS:

The hypothalamic neurosecretory activity of the hypothalamic paraventricular nucleus (PVN)

Morphologically, the hypothalamic paraventricular nucleus (PVN) is part of the anterior group of nuclei, located in the median area of the hypothalamus, which is bilaterally disposed, on both side of the ventricle III.

PVN is composed of large neural areas containing small dense cell populations. In these neural structures, compact areas composed of big magnocellular neurons, voluminous, oval, with nuclei often disposed eccentricsometimes double, with abundant chromatic substance, are located. Nerve cells contain yellow pigment and

Hypothalamic neurosecretory activity study in group C, revealed abnormal neurosecretory activity in both neuronal perikaryon at the magnocellular neurons level and hypothalamic paraventricular nucleus (PVN) as well as along the axonal extensions, similar issues being encountered in neuronal morphology parvocellular areas of periventricular hypothalamic area too. Besides neurons having the neuronal perikarya abundant in neurosecretory granules, there are neurons with a lower neurosecretory activity (Figure 1A).

Exposure of animals to anakinetic stress conditions for 15 days (group S) induced modifications to the PVN neurosecretory activity. In magno and parvocellular areas, there appear neurons whose perikarya are emptied of neurosecretory material. Alongside these, there are neurons with vacuoles with small colloidal grains, representing the neurosecretory products.

pyknotic nuclei, having condensed cytoplasm. Some neurons are hypertrophied with vacuolated cytoplasm. The neurosecretory products are accumulated in the corpus of the axons (Figure 1B).

Galium verum hydroalcoholic extract administration in group E determined the manifestation of moderate to normal neurosecretory activities in about 75% of magnocellular neurons and in those belonging to parvocellular areas of PVN. About 25% of magno and parvocellular neurons are highly active (Figure 1C).

In animals that were treated with *Galium verum* extract and exposed to anakinetic stress (ES group), a widening of the neurosecretory activity in both magnocellular area, as well as in the parvocellular area of PVN (Figure 1D) was emphasized.

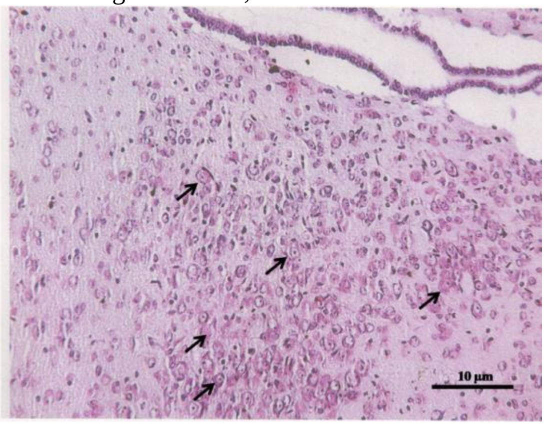

Fig.1A

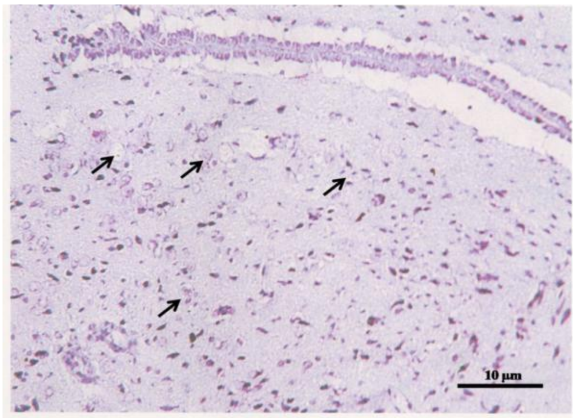

Fig.1B

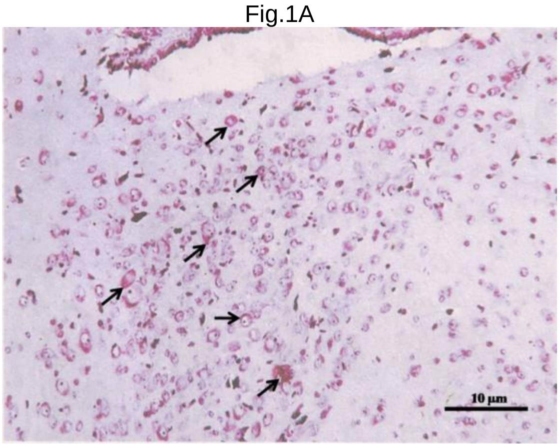

Fig.1C

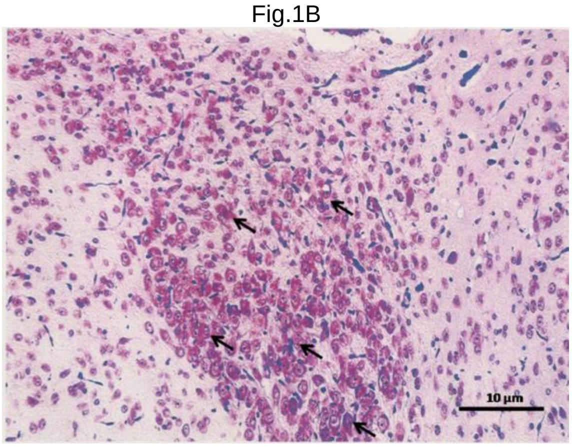

Fig.1D

Figure 1A - D. A - Normal appearance of neurosecretory activity of PVN in group C (× 200), **B** - Decrease of PVN neurosecretory activity, associated with spread of secretion granules along the dendritic axonal extensions, in group S (× 200), **C** - Moderate increase of neurosecretory activity in PVN in group E (× 200), **D** - Intense neurosecretory activity of PVN in group ES, (× 200). Black arrow indicates neurosecretory activity in PVN.

Histopathological study of adenohypophysis

Histological study of the adenohypophysis in group C, showed a normal appearance of the gland, with a characteristic cytoarchitecture, and the main cellular types – acidophilic eritrozinofile darker cells α somatotroph cells (GH cells) with a brick-red coloring and ŋ mammotrophs secretory cells (PRL cells), secrete prolactin, that contain intense red-orange granulations; basophilic β gonadotroph cells (LH - FSH cells) colored in various stains of blue or violet-blue; and the ε corticotroph (ACTH cells) and tireotroph cells (δ cells) colored in azure-blue. The cromophobic cells are uncolored (Figure 2A). Exposure of animals to anakinetic stress (group S), induced significant changes in the appearance of the gland characterized by an increased basophilism associated with a lower, generalized cellularity of the adenohypophysis. A moderate hypoplasia of α acidophilic cells, located in the sides of the gland, was noted. Some cells (both acidophilic and basophilic) are hypertrophied, with an increased cell volume and nucleus volume.

The cytoplasms on this cells are ballooned, containing clear vacuoles of small dimensions. A part of basophilic cells are dystrophic, with pyknotic nuclei, other cells with a round or elongate shape are hypertrophied, intensely vacuolated, with eccentric nuclei (the so - called Crooke cells - characteristic to stress states), amongst the cells clear spaces of different dimensions or connective tissue proliferations being remarked. The sinusoidal capillaries present marked dilatations (Figure 2B).

Galium verum extract administration (group E) produced histological changes at smaller scale, characterized by a slightly increased bazofilism, associated to a moderate intensification of acidophilic character of the gland, too. The presence of intercellular spaces with sparkling look, with acidophilic content, associated with the dilatation of sanguine capillaries lumen (Figure 2C) is also noted. *Galium verum* extract treatment, followed by exposure of animals to anakinetic stress (group ES) resulted in a significant increase in both the acidophilic and basophilic character of the adenohypophysis. The accumulation of secretion compounds in the cytoplasm of acidophilic and basophils cells is remarked. In the glandular parenchyma, there appear much dilated pericellular spaces, vacuolated, full of acidophilic content, the total disappearance of Crooke cells being also remarked (Figure 2D).

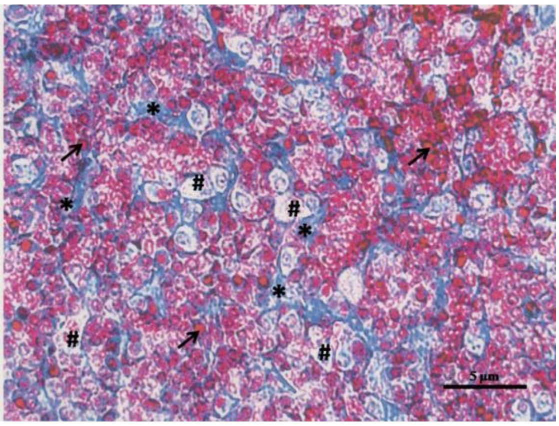
Fig.2A

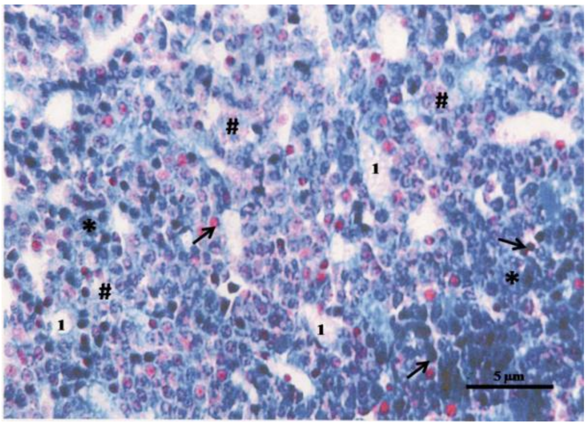
Fig.2B

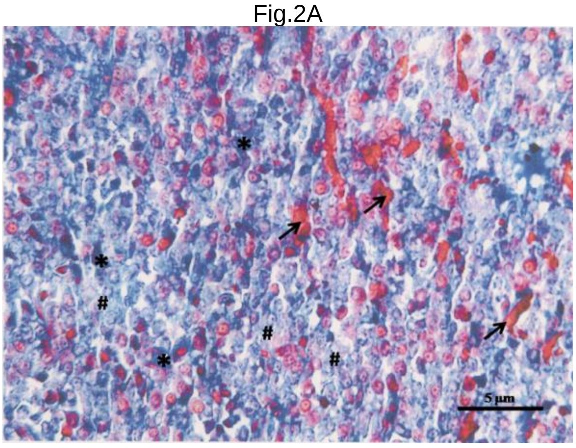
Fig.2C

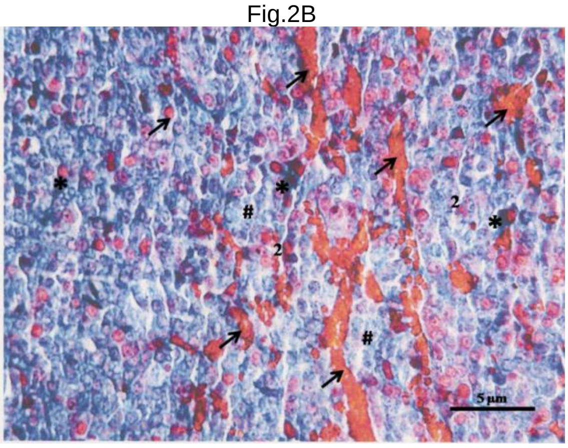
Fig.2D

Fig. 2.A - D. Histopathological appearance of adenohypophysis. **A -** Normal appearance of the adenohypophysis in group C (× 400), **B** -Ggroup S **-** vacuolar appearance, increased basophilism of glandular parenchyma and Crook cells having cytoplasm filled with sparkling content, (× 400), **C** - Group E - slightly increases of basophilism, moderate intensification of adenohypophysis acidophilic character, (× 400), **D** - group ES - significant increase in the basophilic and acidophilic character of the adenohypophysis, (× 400). Black arrow = acidphiles cells; * = basophile cells; # = cromophobe cells; 1 – vacuolation areas; 2 – dilatation of sinusoides.

Histological study of adrenals

The histological examinations have emphasised a normal aspect of the adrenals in group C, with the 3 constituting layers of the cortex (the glomerulosa, fasciculata and reticularis layers) being well delimited and visible (figure 3A). The cortex zone represents 80 - 90% of the adrenal gland's total volume.

Zona glomerulosa (mineralocorticoid hormone synthesis place) consists of small columnar cells, closely packed, lighter stained. The cytoplasm has a slightly acidophilic character, the spherical nuclei being located in the center of the cell. Zona fasciculata consists of large polyhedral cells, arranged in vertical colums. Among the cell cords, the presence of capillaries can be remarked. The spongiocytes secrete glucocorticoid hormones that affect carbohydrate and protidic metabolism and exert a strong influence on fat storage. Zona reticularis – is the area of 17 - ketosteroids hormone synthesis, is composed of small, polyhedral cells, grouped into clumps and colums which intersect in various ways to form a crosslinked network around capillaries.

Adrenal medulla also has a normal appearance. Glandular parenchyma consists of cortical cells structured in the shape of irregular cords. Among the cortical cords, lymphoid tissue cords can be highlighted, which include the islands of medulla cells. They are made up of smaller cells, having a basophilic character.

In S group there is a moderate thickening of the cortex, particularly the zona fasciculata, this aspect being associated with a decrease in medullary thickness, the ratio cortical / medullary being slightly in favour of the cortical (Figure 3B).

In the zona fasciculata, one can observe the disturbance of the arrangement of parallel cords of cells. At the limit between the zona fasciculata and glomerulosa, some spongiocytes are increased in volume, atrophic, with hypertrophied nuclei.

Between the spongiocyte cellular cords of the zona fasciculata, large groups of inactive cells are inserted, with a dark compact appearance (so called "dark cells"), characteristic to stress states. These cells, many of which have lost their spongiocyte character, present a condensed cytoplasm, hypercrome, with pyknotic nuclei, intensely colored.

The capillaries of the zona fasciculata, and especially of the zona reticularis, are heavily dilated. Chromaffin cells of the adrenal medulla are hypertrophied, being grouped in cell cords, separated by wide lacunar areas.

Galium verum extract administration (group E) did not induce significant changes in the morphology of the adrenal glands. It can be noted, however, a thickening trend of the zona fasciculata, the cortical / medullary ratio being in favor of cortical. In the zona fasciculata and especially in the reticularis adjacent one, there appear groups of active cells, with compact cytoplasms, lacking the spongiocyte character. Adrenal medulla, lower in volume, is formed of compact cell cords, having small, lacunar areas occurring through (Figure 3C).

Galium verum hydroalcoholic extract administration in animals exposed to anakinetic stress (group ES) induced significant changes in the morphology of the adrenal glands, being primarily characterized by a thickening of the cortex. In zona fasciculata, the parallel cords cells no longer have the normal, characteristic spongiocitar appearance. The cytoplasms of most of the cells are lacking of lipid inclusions, having spherical, clearly defined nuclei.

Medulla is well developed, consisting of cellular cords separated by large areas where connective tissue includes intense dilated sinusoidal capillaries (Figure 3D).

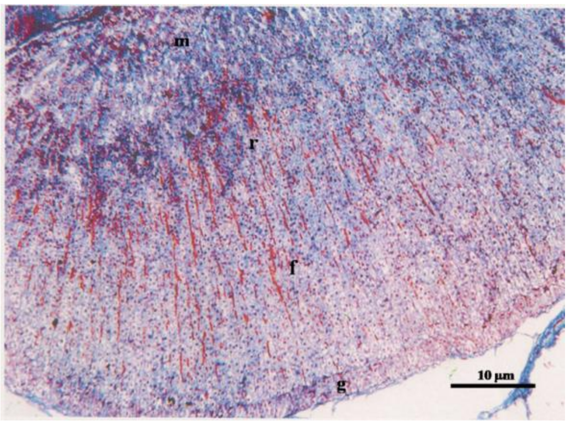

Fig.3A

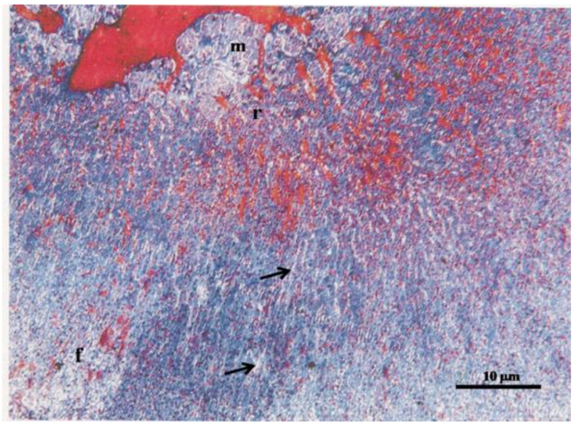

Fig.3B

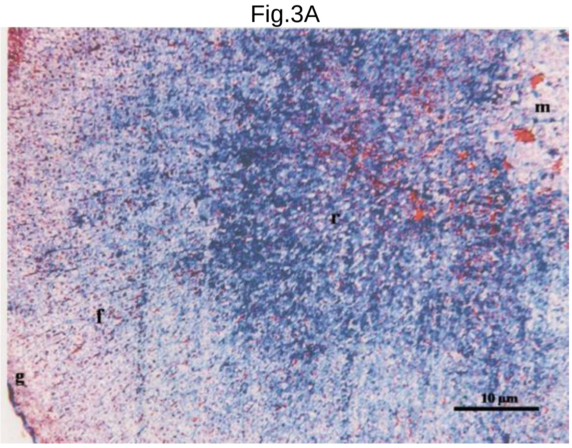

Fig.3C

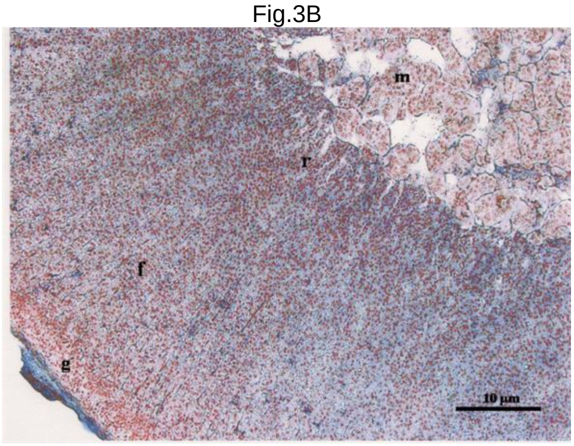

Fig.3D

Fig. 3.A - D. Histopathological appearance of adrenals. **A -** Normal appearance of adrenal morphology in group C, (× 100), **B** – Zona fasciculata of group S - so-called "dark cells" and marked dilatation of capillary lumen. The medulla is highly vacuolated, (× 100), **C** - Zona fasciculata of group E - active cell groups with compact cytoplasm, devoid of spongiocyte character. Medulla is reduced in volume, (× 100), **D** - Zona fasciculata of group ES - cells with cytoplasm lacking of lipid inclusions (× 100). G - zona glomerulosa; f - zona fasciculata; r - zona reticulata; black arrow = dilatation of sinusoides.

DISCUSSION:

HPA axis comprises an assembly of stress responses mediated by the brain, pituitary and adrenal glands. First, the hypothalamus releases a compound called corticotropin releasing factor (CRF), which triggers the release of adrenocorticotropic hormone (ACTH). ACTH is released into the blood, causing the adrenal cortex to secret corticosteroid hormones, especially cortisol. Cortisol increases the availability of refueling the body (carbohydrates, fats and glucose) with substances necessary for the body's response to stress (Dornhorst, 1981).

Morphologically, the hypothalamus, which is the upper floor of HPA axis, is a nervous structure of gray matter, composed of large neural areas, representing the hypothalamic nuclei. Nerve cells that are included in hypothalamic nuclei contain yellow pigment and vacuoles with small colloidal granulations, which represent the neurosecretory products.

Hypothalamic neuropeptides are releasing factors that control the activity of the adenohypophysis, respectively, biosynthesis and secretion of adenohypophysis hormones (adrenocorticotropic hormone - ACTH; gonadotropic hormones - FSH and LH, prolactin-stimulating hormone - TSH, somatotropic hormone - STH etc.) or inhibitory secretion in different physiological conditions.

Among the hypothalamic nuclei, the hypothalamic paraventricular nucleus (PVN) is part of the anterior group of nuclei situated near the median area of the hypothalamus, that are bilateral disposed on both sides of the ventricle III. Inside the PVN, there occur the biosynthesis and the release of several neuropeptides and a variety of neurotransmitters. PVN contains magnocellular neurons and a few groups of small neurons, called parvocellular neurons (Levin and Sawchenko, 1993; Page, 1988). PVN is also involved in a variety of behavioral activities, as well as the organization of the autonomic and endocrine response to stress conditions (Herman and Cullinan, 1997; Nayanatara *et al.,* 2012; Puică *et al.,* 2004).

The study of neurosecretory activity in magnocellular and parvocellular hypothalamic system provides important data on the biosynthesis and release of neuropeptides like vasopressin and oxytocin (in PVN and NSO), as well as the neurohormones released from the parvocellular hypothalamic system, where the secretion and release of release factors (releasing factors - RF) takes place, through which the coordination of the secretory activity of the adenohypophysis occurs - median floor of HPA axis (Exarcu, 1981; Herman and Cullian, 1997; Nayanatara *et al.,* 2012; Page, 1988; Popoviciu and Hăulică, 1982).

The secretory activity of the adenohypophysis coordinates the biosynthesis and secretion of thyroid hormone, genital glands and the adrenal glands - target organs of HPA axis (Dancăşiu, 1974; Roman and Puică, 2013). Schematically, the control mechanism of secretory activity of the adrenal axis HPA is the following: CRH is released in the hypothalamus. In response to CRH stimulation, the adenohypophysis corticotropes synthesize and release the adrenocorticotropic hormone ACTH. When ACTH reaches the systemic circulation, it binds and activate the receptors on the surface of the adrenal cortex cells. The receptors of the adrenocortical cells synthesise glucorticoids, as a response to activation (Cvijić and Đorđević, 2003; Puică *et al.*, 2008; Swanson *et al.*, 1986).

Our results showed different structural reactivity of the HPA axis in the three experimental groups. Thus, in group S - subject to anakinetic subchronic stress, it was noticed a moderate decrease in PVN neurosecretory activity, also associated with moderate structural changes of the adenohypophysis and adrenal glands. The data suggests lower levels of hypothalamic corticoliberin (CRH), of adenohypophysis ACTH secretion, as well as of steroid hormone secreted by the adrenal, facts which influenced less the dynamics of body weight during the experiment. Single administration of the *Galium verum* extract in group E did not significantly affect the activity of HPA axis. In contrast, the *Galium verum* extract treatment, associated with stress exposure in ES group, determined an increase in PVN neurosecretory activity - related to a possible stimulation of hypothalamic CRH release.

Whereas in this experimental group the most striking morphological changes were observed in the paraventricular hypothalamic area, where, among other neurohormones, some of the factors that control the activity of the adenohypophysis are released, the stimulation of the neurosecretory activity, respectively the increased release of hypothalamic neuroliberines, reverberated on the morphology and functions of both the pituitary as well as the adrenals.

Structural aspects observed in the adenohypophysis and adrenals suggest both a possible activation of adenohypophysis hormone secretion, as well as the stimulation of steroid hormones secretion by the adrenal. We note again that in groups E and S the reactivity of HPA axis oscillated closer to normal limits. The increased activity of the HPA axis suggests the existence of a positive synergistic mutual effect between the two analyzed factors: plant extract and exposure to stress.

We believe that exposure to anakinetic stress induced a stimulatory effect of potentiation of the bioactive compounds action contained in the plant extract of *Galium verum* on the morphology and function of the entire set of analyzed parameters.

Exposure of animals to subchronic anakinetic stress decreases the PVN neurosecretory activity, suggesting the decrease of hypothalamic CRH secretion. Following treatment of animals with the vegetable extract and exposure to anakinetic stress (group ES), there was noticed an increase in PVN neurosecretory activity, suggesting an increase of corticoliberin release at hypothalamic level. Exposure of the animals to anakinetic stress resulted in increased number of basophyl cell, suggesting a decrease of adenohypophysis hormones secretion. *Galium verum* extract administration produced smaller changes in the gland morphology, suggesting slight increase of the secretion of adenohypophysis hormones. *Galium verum* extract administration associated with the exposure of animals to anakinetic stress caused a significantly increase in acidophilic and basophilic character of the adenohypophysis, this aspect being possibly associated with increased secretion of adenohypophysis hormones. Immobilization stress induced changes at adrenal level, suggesting a reduced secretion of glucocorticoid hormones. *Galium verum* hydroalcoholic extract administration in animals exposed to anakinetic stress induced morphological changes that prove an increase in adrenal glucocorticoid hormone synthesis.

CONCLUSIONS:

The HPA axis histological study showed that administration of the 25 mg *Galium verum* extract/100 g bw in conditions of animals exposure to anakinetic stress induced important morphological changes of the entire hypothalamic-pituitary-adrenal axis constituent assembly, results which confirmed stimulation of its secretory activity.

ACKNOWLEDGEMENTS:

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