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Potential *in vivo* immunomodulatory effects of *Astragalus membranaceus, Iris germanica, Glycyrrhiza glabra* and its active components on mice after exposure to bisphenol S

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ABSTRACT

Objective: This study aims to examine the effects of *Astragalus membranaceus*, *Iris germanica*, and *Glycyrrhiza glabra* extracts on immune system regulation in mice. The use of BPS as a replacement for other bisphenols has prompted scientific attention in them toxicological evaluations in vivo and in vitro. Given the strong association between bisphenol S (BPS) and immune-related disorders, we investigated whether BPS can cause damage in the spleen of a mouse model. ***Approaches:*** We obtained male CD1 mice weighing between 33 and 39 grams and aged 8 weeks obtained from the Cairo Serum and Vaccine Institute (Egypt). The mice were housed in sterile, individually ventilated cages with a 12-hour light/dark cycle. The environment was maintained at 22 °C with 45% relative humidity. There were six groups of mice, and seven mice were in each group. One group was given olive oil (control group), while the remaining five groups received BPS at a dose of 100 µg/kg body weight/day. We administered the medication by intragastric injection for 55 consecutive days. Three types of herbs were used for treatment, with one group showing a positive effect. We administered a daily dose of 200 mg/kg of *Astragalus membranaceus* extract to Group 1 (G1). Group 2 (G2) received the same dose of *Glycyrrhizin glabra* extract. Group 3 (G3) received 200 mg/kg/day of *Iris germanica* extract. Group 4 (G4) received a mixture of herbal extracts in equal proportions (1:1:1 v/v), with a total dose of 200 mg/kg/day. We collected spleen tissue samples from the control and treatment groups. ***Results:*** We compared the positive control group with its negative counterpart, and then compared the herb-treated groups with the infected group. The individual use of the herbs. However, the group receiving the herbal mixture demonstrated the most significant improvement and performed most effectively. *Astragalus membranaceus*, *Iris germanica*, and *Glycyrrhiza glabra* extracts enhance the immune-modulating effects of the spleen by preventing harmful substances from entering the body.

Keywords: *Astragalus membranaceus*, *Iris germanica*, *Glycyrrhiza glabra*, spleen, immunomodulatory effects, Biphenyl S.

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INTRODUCTION

Several industries and production processes widely utilize bisphenol A (BPA), known for its endocrine-disruptive properties. Due to increasing concerns regarding toxicology, there is ongoing research on the use of BPA in food and beverage contact applications. The manufacturing of epoxy resins and polycarbonates is substituting Bisphenol S (BPS) for BPA. Researchers have identified BPS in environmental and human samples such as water, soil, sediments, human urine, and blood **(Akash et al., 2023)**. Studies on animals and in epidemiology have shown that exposure to BPA leads to several negative consequences, including impacts on the immune system and metabolic processes **(Naomi et al., 2022)**. Research conducted in laboratory settings (*in vitro*) or on living organisms (*in vivo*) has established a strong link between exposure to BPS and harmful effects on health. Like BPA, BPS also exhibited hormonal effects, including estrogenic and androgenic activities, as well as antiestrogenic and anti-androgenic activities **(Park et al., 2022)**. According to **Wu et al. (2023)**, 8-hydroxy-2′-deoxyguanosine was found in human urine, which shows that BPS exposure led to oxidative stress. The exposure to BPS resulted in DNA damage, as demonstrated by **Liao et al.** in **2022**. **Li et al.** reported in **2023** that it also impacted the signaling system associated with lipid metabolism.

Several studies have examined whether or not exposure to BPA and its replacements might impact immunological responses and function, as they have the potential to affect endogenous estrogen signaling through similar activation pathways as endogenous estrogens **(Faheem and Bhandari, 2021)**. **Kodila et al.'s 2023** study found that BPA exposure triggers the production of pro-inflammatory cytokines, causing immunological dysfunction in the modulation of innate immunity, control of T cells' functionality, lymphocyte proliferation, and antibody responses. Many people widely acknowledge the spleen as a fundamental location for the start of immune responses. It performs a crucial role in immunological responses, including the reaction to infection and the removal of antigens and cell debris from the bloodstream **(Mukherjee et al., 2023)**. **Al-Griw et al. (2023)** found that BPA induces mitochondrial dysfunction in mouse splenocytes by elevating oxidative stress levels. **Zhao et al.** discovered that exposure to BPA affected the functioning of immunomodulation and hematopoiesis in mice's spleens. The findings also revealed that exposure to BPA in **2020** resulted in changes to the concentration and sex-specific cellular and microanatomical components of the spleen.

The immune system serves as a barrier against outside chemicals and is crucial for maintaining the body's internal balance, known as homeostasis **(Burgos-Aceves et al., 2021)**. We can classify immunotoxicity, or the detrimental effects on the immune system, into two categories: immunosuppression and immunoenhancement. These effects might manifest as hypersensitivity reactions such as allergies and autoimmune diseases **(DeWitt et al., 2019)**. The spleen and thymus are organs composed of lymphoid tissue that play a crucial role in the immunological response to antigens that pose a danger to the immune system. Xenobiotics can cause immunotoxicity to the spleen, which is responsible for producing and presenting lymphocytes **(Semwal et al., 2022)**. Despite being active throughout the prenatal and postnatal periods, the thymus continues to produce and mature new T cells throughout a person's whole life **(Miller, 2020)**.

The typical healthy adult's spleen weighs approximately 200 g and is located in the left region of the belly. One may perceive the spleen as a dual organ. The organ's job is to clean the blood by getting rid of abnormal cells like old and broken red blood cells. It also makes

antibodies and lymphocytes, which are immune system cells that fight disease **(Ouyang et al., 2021)**. The spleen is the most prominent lymphoid organ. The spleen contains roughly 25% of the total lymphocytes found in the body. The spleen is a vital component of the entire immune system. The spleen primarily contains T lymphocytes, B lymphocytes, and natural killer (NK) cells, which are the major immune cells present in this organ. The immune cells and components in the spleen perform non-specific immunological duties through phagocytosis.

Additionally, T and B lymphocytes mediate specific immune functions through cellular and humoral immunity **(Ahrorova, 2021)**. The spleen also contributes to the immune response by regulating the distribution of immune cells in both the spleen itself and in the bloodstream. Lymphocytes are a category of immune cells found in the bloodstream, consisting of T and B lymphocytes. B lymphocytes are the primary cells responsible for the humoral immune response. Natural killer (NK) cells are the primary agents of the innate immune response, responsible for carrying out innate immunological reactions. They do not depend on antibodies or complements but have the ability to directly eliminate target cells, thereby contributing to infection defense and immune system regulation and surveillance **(Aliyu et al., 2021)**.

The spleen, which houses around 25% of the body's circulating T cells, is the biggest immunological organ and serves as the central hub for immune activity. It actively engages in cellular immunity and controls the distribution of T lymphocyte subsets in the bloodstream. **Suttorp and Classen (2021)** have reported no known pathological alterations in the spleen during autoimmunity, despite the common association of AIH with the spleen.

Plants are the primary reservoir of bioactive compounds used in illness treatment. According to the World Health Organization (WHO), a significant proportion of the global population continues to depend on plant-based treatments for primary healthcare, despite the existence of synthetic medications **(Aware et al., 2022)**. The process of drug development heavily relies on medicinal plants, and the historical use of these plants has inspired several contemporary medications. Although molecular modeling has made significant breakthroughs, medicinal plants remain a critical source for the discovery of novel medications and potential therapeutic candidates. Therefore, when it comes to treating diseases where medication therapy is a logical strategy, plant materials are valid substances to begin the search for novel agents **(Chaachouay and Zidane, 2024)**.

Astragalus membranaceus, often known as *A. membranaceus*, is well recognized as one of the most commonly used herbal remedies on a global scale. Traditional Chinese medicine employs this substance not only as a medicinal remedy but also as a dietary source to enhance the functioning of the spleen and restore vitality. *Astragalus* polysaccharide (APS) is a bioactive heteropolysaccharide that is soluble in water. The stems or dried roots of *A. membranaceus* serve as its source. The parts are complicated and have many different properties. The monosaccharides are mostly linked by a-type glycosidic bonds to the polymeric carbohydrates **(Zheng et al., 2020 a)**. *Astragalus* polysaccharides, or APS, is the primary bioactive compound present in *A. membranaceus*. It possesses a wide range of pharmacological properties, as demonstrated by **Peng et al.** in **2023**. **Li et al. (2022)** have extensively employed APS due to its low toxicity and side effects, lack of residue, and absence of tolerance.

Polysaccharides are large molecules made up of smaller units called monosaccharides. Nature has discovered over 300 different types of polysaccharide compounds. *Astragalus membranaceus*, a traditional Chinese medicinal herb, contains *Astragalus* polysaccharide (APS) as its primary active constituent. Glucose, rhamnose, galactose, arabinose, xylose, mannose, glucuronic acid, and galacturonic acid make up APS **(Chen et al., 2023)**. We can divide APS into two components based on its molecular weight: APS-I, which has a molecular weight above

500 kDa, and APS-II, which has a molecular weight below 10 kDa. It has several pharmacological actions, such as immune function control, anticancer activity, anti-fibrotic properties, blood sugar reduction, blood lipid lowering, and antibacterial and antiviral activities **(Li et al., 2023)**.

The structural characteristics of *A. membranaceus* polysaccharide (APS) are responsible for its immunomodulatory actions. APS has a molecular weight range of 8.7–4800 kDa, which consists of different combinations of monosaccharide compositions. The APS compound is immunomodulatory and has a chemical structure consisting of a dextran backbone with branches connected every 10 residues. The molecular weight of APS is around 21 kDa, as reported by **Dong et al.** in **2023**.

The world is home to the *Iris* plant, a member of the Iridaceae family. The genus consists of over 300 species that are well-known for their decorative significance and therapeutic properties. Studies have demonstrated the significant therapeutic potential of *Iris* genus species in treating pulmonary asthma, cancer, inflammation, liver illnesses, and uterine problems **(Joshi, 2023)**. Lots of study into the chemicals found in different types of *Iris* has led to the discovery and extraction of many chemicals, including quinones, triterpenoids, flavonoids, isoflavonoids, and stilbene glycosides **(Jat et al., 2022)**. Humans ingest flavonoids and isoflavonoids, significant plant compounds with a wide range of structures, as part of their diet. Consuming a diet high in isoflavones has been shown to decrease the likelihood of developing cancer, namely breast and prostate cancer **(Pejčić et al., 2023)**. It has been shown that isoflavones play a role in cancer, osteoporosis, heart disease, and menopause symptoms. They also have estrogenic, antimicrobial, anti-inflammatory, and antioxidant effects **(Aboushanab et al., 2022)**.

Several industries, including cosmetics, pharmaceuticals, and the food business, continue to utilize *Iris* species today. Morocco uses the rhizomes of *Iris* species, also known as Orris roots, as a constituent in Ras el Hanout, a Moroccan spice mix **(Khatib et al., 2022)**. Similarly, people strip the rhizomes of *I. germanica* L. of their outer layer and use them as a seasoning in ice cream, confectionery, baked goods, and alcoholic drinks **(Crişan et al. 2019)**. In southern Europe commercial growers cultivate *Iris* species to produce tooth powder, toothpaste, and teething rings. Additionally, high-end luxury perfumes and lotions, such as "Iris Ganach" by Guerlain, "Extravagance d'Amarige" by Givenchy, "Chanel 19" by Cartier, and "So Pretty" by Cartier, currently employ certain *Iris* species, such as *I. florentina* L. and *I. germanica* L. **(Xie et al. 2017; Mykhailenko, 2018; Amin et al. 2021)**.

Scientists have recently found many new bioactive chemicals from many families in *Iris* species. These include alkaloids, flavonoids and their derivatives, quinones, terpenes, steroids, and simple phenolics **(Okba et al., 2022)**. Recent pharmacological research has found that these chemicals have notable impacts on human health, including qualities that help prevent cancer and combat cancer, as well as antioxidant, antiplasmodial, immunomodulatory, and anti-inflammatory actions **(Siddiqui et al., 2022)**.

Glycyrrhiza glabra is a perennial shrub that is highly resilient and may grow up to a height of 2.5 meters. The leaves are compound, imparipinnate, alternating, and consist of 4–7 pairs of oblong, elliptical, or lanceolate leaflets. The flowers are slender, usually papilionaceous, arranged in axillary spikes, and have a lavender to violet hue. Abbreviated, bell-shaped, and adorned with glandular trichomes, the calyx has lanceolate apices. The fruit is a compacted legume or pod, measuring up to 1.5 cm in length. It stands upright, is smooth, and has a

somewhat net-like pattern on its surface. Typically, it has 3–5 kidney-shaped brown seeds. Around 1.5 cm in length, the taproot splits into 3–5 subsidiary roots **(Lohar et al., 2020)**.

Sugars, proteins, amino acids, vitamins (B1, B2, B3, B5, E, and C), bitters, resins, alkaloids, glycosides, flavonoids, phenolics, saponins, tannins, terpenes, essential oils, steroids, gums, and mineral salts are among the substances that make up licorice **(Sharma et al. 2018)**. The herb most widely utilized is licorice. It serves as the primary component in about 60% of traditional Chinese medicine (TCM) medicines **(He et al., 2023).** The leaves are pinnate, measuring 15 cm in length, compound, and arranged in four to seven pairs of oblong shapes with a smooth surface. The flowers are slender and stalkless, measuring 0.8–1.2 cm in length. Spikes that emerge from the leaf axils arrange the lavender to violet colored flowers. The inflorescence is loosely structured. The fruit is a compact legume or pod, upright, smooth, and measures 2-3 cm in length. It contains 3–8 kidney-shaped seeds with a dark green color and a smooth surface. The seeds are around 2 mm in diameter. The roots have well-developed characteristics, including a taproot structure that is horizontal, woody, and stoloniferous. Moreover, the taproot divides into three to five subsidiary roots. The roots and rhizomes exhibit a color range from brown-green to dark brown, with the ability to penetrate up to a depth of 1 meter **(Husain et al., 2021)**.

According to the chemical analysis, licorice roots make up 50% of their weight. This is made up of water-soluble metabolites and sugars (5–15% glucose, sucrose, and mannitol), starch (25–30%), 3–10% D-glucose, glycyrrhizin (10–16%), and amines (1–2% asparagine, betaine, and choline). Additionally, licorice extract contains significant amounts of sterols (stigmasterol and β-sitosterol) **(Lim, 2016)**. Different parts of plants (roots, stems, and leaves) naturally make different amounts and types of these compounds. These differences depend on things like the climate where the plants were grown, when they were harvested, how they were stored, processed, and how they were separated **(Rizzato et al., 2017)**. **Wahab et al. (2021)** found that the roots of *G. glabra* contain 9.7% total polysaccharides, of which around 1.6% are water-soluble polysaccharides composed of rhamnose, arabinose, mannose, glucose, and galactose.

Using animal models, this study aims to demonstrate the extent of the effects of extracts from *Astragalus membranaceus*, *Glycyrrhiza glabra*, and *Iris germanica*, as well as their bioactive compounds, on the immune system and immune markers.

MATERIALS AND METHODS

Animal-assisted therapy and bio-psychosocial treatment

The study used male CD1 mice (33–39 g body weight, 8 weeks of age) purchased from the Serum and Vaccine Center in Cairo, Egypt. We housing the mice in sterile, individually ventilated cages under controlled conditions, with a 12-hour light/dark cycle at a temperature of 22 °C and a relative humidity of 45% **(Ortega-Saez et al., 2023)**. The researchers gave intragastric administration to six groups of mice, each composed of seven mice, over a period of 55 consecutive days. One group served as a control and received olive oil, while the other five groups were administered 100 μg/kg/day of BPS. The other four groups were treated with different herbal preparations: Group 1 received a daily dose of 200 mg/kg of *Astragalus membranaceus*, Group 2 received a daily dose of 200 mg/kg of *Glycyrrhiza glabra*, Group 3 received a daily dose of 200 mg/kg of *Iris germanica*, and Group 4 received a daily dose of a mixture of herbs. Spleen tissues were collected from both the control and BPS-treated groups for histological assessment **(Zhao and Cai, 2023)**.

Extraction

We obtained the Astragalus membranaceus powder from the National Research Center in Dokki, Giza, Cairo, Egypt. We ground 500 grams of dried roots from *A. membranaceus* into a powder and then soaked them in a 50% hydroalcoholic solution for 48 hours, a process known as exhaustive maceration. We repeated this process three times, each time using 2.5 liters of the solution. The solvent was removed under vacuum, resulting in approximately 68 g of dehydrated hydroalcoholic extract. The filtered extract was mixed and concentrated using rotary evaporation to eliminate ethanol. The concentrated extract was then freeze-dried with a Virtis Freeze Dryer from the Virtis Company, located in New York, USA. We stored the A. membranaceus powder, which resulted in a yield of 31.27%, at a temperature of 4 °C for subsequent experimentation **(Santoro et al., 2020)**.

We pulverized the air-dried rhizomes of *Iris germanica* and passed them through a 40-mesh filter. We then subjected them to a cold extraction using alcohol. A macerator with 70% ethanol extracted a measured amount of 500 g of the powdered substance. A rotating vacuum evaporator under low pressure condensed the hydroalcoholic extract. We used a liquid-liquid extraction procedure to fractionate the hydroalcoholic extract after drying it. This included utilizing several organic solvents, including hexane, DCM, ethyl acetate, and butanol, in a sequence of increasing polarity. We dehydrated the fractions and then stored them in hermetically sealed glass containers at a temperature of 4 °C for future use **(Farooq et al., 2023)**. This research was conducted at the National Research Center located in Dokki, Giza, and Cairo, Egypt.

We dried the root of *Glycyrrhiza glabra* (licorice) in the sun for two days and then ground it into a fine powder using a mechanical grinder. A solution of licorice root in ethanol was made by combining 30 grams of powdered licorice root with 150 milliliters of ethyl alcohol that had a concentration of 70% (w/v). The mixture was placed in a flask and gently shaken for a period of 7 days. Subsequently, the ethanolic extract was initially filtered using a muslin cloth to remove any large particles, and then filtered again using Whatman no. 1 filter paper. The solution was thereafter stored in a hermetically sealed container of amber hue and maintained at a temperature of 4°C for future utilization **(Malvania et al., 2019)**. The experiment was conducted in the Chemistry Laboratory of the Faculty of Home Economics, Al-Azhar University, Tanta, Egypt.

Quantifying the overall phenolic and flavonoid concentrations

We used the Folin-Ciocalteau technique **(Singleton & Rossi, 1965)** to quantify the total phenolic content of *Astragalus membranaceus*. In summary, a 0.1 mL sample was combined with a 1 mL diluted ten-fold Folin-Ciocalteau reagent and left to incubate at room temperature for 5 minutes. Following this, a 1 mL solution of Na_2Co_3 at a concentration of 0.1 g/mL was added to the combination. The optical density was determined at a wavelength of 765 nm following a 90-minute incubation period at ambient temperature. The findings were reported as milligrams of gallic acid equivalents per gram (mg GAE/g). The samples' total flavonoid content was assessed using a colorimetric technique, as described by **Zhishen et al. (1999)**, with some adjustments. The 0.1 mL sample was combined with 0.3 mL of a $NaNo_2$ solution with a concentration of 0.05 g/mL in a test tube. The mixture was then left to incubate for 5 minutes. After that, 0.3 mL of an $AlCl_3$ solution with a concentration of 0.1 g/mL was added and the mixture was incubated for an additional 6 minutes. The process was halted by introducing 2 mL of a 1 mol/L NaOH solution. The absorbance of the combination was promptly measured at a

wavelength of 510 nm. The total amount of flavonoids was measured and reported as rutin equivalents (mg RE/g).

We used the Foline-Ciocalteau technique, a colorimetric test for phenols, to quantify the phenolic compounds in the methanolic extract of *Glycyrrhiza glabra*. A calibration curve using the standard "gallic acid" was created to determine the total phenolic content in the sample. The total phenolic content in the root extract is expressed as Gallic acid equivalent (GAE) and measured in milligrams per gram of dry mass **(Lin and Tang, 2007; Alhakmani et al., 2013)**. The total flavonoid levels of the methanolic extract of roots were measured using the Aluminum chloride colorimetric technique using a twin beam UV spectrophotometer. A calibration curve using the standard "Quercetin" was created to determine the total flavonoid concentration in the sample **(Ahmad et al., 2014)**. The flavonoid levels in the sample of root extract are quantified and expressed as Quercetin equivalent, measured in micrograms per gram of dry mass.

The researchers in the study conducted a spectrophotometric approach to determine the total phenolic contents of *Iris germanica*, as described by **Kim et al.** in **2003**. Briefly, the researchers mixed 1 ml of the extract with the appropriate solvents and then combined it with 9 ml of deionized distilled water. One milliliter of Foline-Ciocalteau's phenol reagent (FCR) was added to the mixture while shaking continuously. After a duration of 5 minutes, a volume of 10 milliliters of a solution containing 7% sodium carbonate (Na_2Co_3) was introduced and thoroughly blended. The solution was diluted to a volume of 25 ml using deionized distilled water and properly mixed. The absorbance at 750 nm was measured after 90 minutes at a temperature of 23°C, with deionized distilled water used as a blank. A standard curve for total phenolics was generated by employing a gallic acid standard solution (ranging from 0 to 100 mg/L) following the previously described approach. The total phenolics were quantified by measuring the amount of Gallic acid equivalents (GAE) in milligrams per gram of the dried sample. The total flavonoid contents were determined by using the technique outlined by **Park et al. (2008)**. In this protocol, a total of 0.3 ml of extracts, 3.4 ml of 30% methanol, 0.15 ml of $NaNo_2$ (0.5 M), and 0.15 ml of $AlCl_3.6H_2O$ (0.3 M) were combined in a 10 ml test tube. Following a period of 5 minutes, a volume of 1 milliliter of sodium hydroxide (1 molar) was introduced. The solution was quantified at a wavelength of 506 nm. A standard curve was generated for the total flavonoids by utilizing a rutin standard solution (ranging from 0 to 100 mg/L) following the identical process mentioned before. The total flavonoids were quantified by measuring the amount of rutin equivalents in each gram of dried material.

Methods for obtaining blood samples, isolating serum, and preparing tissue samples

Each animal's retro-orbital Venus plexus provided blood samples, which we separated into two halves 24 hours after the previous treatment. The initial portion was gathered and placed in sterile, dry tubes containing EDTA for the purpose of conducting an erythrogram and leukogram. This includes the measurement of various parameters such as red blood cell count (RBCs), hemoglobin concentration (Hb), packed corpuscular volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total white blood cell count (WBCs), and platelets (PLTs). Simultaneously, the second portion of the blood was gathered into unadorned tubes to facilitate the separation of serum. The blood was then left to solidify at ambient temperature for a duration of 20 minutes, after which it was subjected to centrifugation at a speed of 5,000 revolutions per minute for a period of 10 minutes. The individual serums were meticulously collected and preserved in a deep freeze at a temperature of -20°C for the purpose of conducting biochemical experiments **(Al-Shammari et al., 2024)**.

Under light anesthesia, we killed the rats by cervical decapitation, administering a ketamine-xylazine combination at a dosage of 0.15 ml per 100 grams of body weight intraperitoneally. A surgical procedure was conducted to make an incision in the middle of the abdomen in order to expose the internal organs. The spleen of each rat was surgically removed, separated from the surrounding tissues, rinsed with normal saline, and subsequently processed for histological and immunohistochemical evaluations. The spleen samples were preserved in 10% neutral buffered formalin for histological and immunohistochemical analyses **(Emam et al., 2023)**.

The variables being measured are the body weight and the index of immunological organs

It is important to regularly monitor the overall health conditions of mice during the trial, paying particular attention to their look, level of activity, and eating behavior. At intervals of 0, 5, 11, and 15 days, the weight of each group of mice was measured, and the data was documented. The spleen and thymus were promptly removed using surgical means and their weights were measured **(Fei et al., 2022)**. The formula used to determine the immunological organ index is as follows: The spleen/thymus index (mg/g) is calculated by dividing the weight of the spleen or thymus (in milligrams) by the body weight (in grams).

The cytotoxicity of natural killer (NK) cells in splenocytes was assessed

We conducted the natural killer cell activity assay using the previously published method, with some adjustments. In summary, splenocytes effector cells were cultured with YAC-1 target cells, blank medium, or 1% NP-40 in a 96-well plate for 4 hours at 37 °C in a humidified atmosphere of 5% CO_2. The ratio of target cells to effector cells was 1:50. Following centrifugation at 400×g for 5 minutes, the LDH activity in the supernatant was assessed using the LDH Cytotoxicity Detection Kit (ThermoFisher Scientific Inc., USA) as per the manufacturer's instructions **(Chen et al., 2020)**. The activity of NK cells, expressed as a percentage, was determined by calculating the absorbance observed at 490 nm using the following equation: The activity of NK cells, expressed as a percentage, is calculated using the formula: NK cell activity (%) = (Absorbance of the sample minus Absorbance of the control) divided by (Absorbance of the NP40 - Absorbance of the control), multiplied by 100%.

An analysis of serum immunoglobulins

We used ELISA test kits (MM-090502, MM-040302, and MM-040202, MeiMian, China) to determine the levels of serum immunoglobulins IgA, IgE, IgG, and IgM, following the manufacturer's instructions. Concisely, the regular sample or serum diluted sample was introduced to the enzyme plate and kept at a temperature of 37℃ for a duration of 30 minutes. After washing the enzyme plate five times, the HRP-conjugate reagent was applied and incubated at a temperature of 37℃ for a duration of 30 minutes. Subsequently, chromogen solutions A and B were introduced to the enzyme plate and subjected to incubation at a temperature of 37℃ for a duration of 30 minutes, following a thorough washing process consisting of 5 repetitions. After the stop solution was added, the absorbance was measured at a wavelength of 450 nm within a time frame of 15 minutes **(Yin et al., 2022)**.

The blood levels of IL-1, TNF-, IL-6, and IL-12p70 were quantified

The levels of serum IL-1β, TNF-α, IL-6, and IL-12 p70 were determined using enzyme-linked immunosorbent assay (ELISA) kits from Bioss, Beijing, China, following the manufacturer's instructions. The plasma endotoxin was quantified using the Bioendo Limulus Amoebocyte Lysate (LAL) Chromogenic Endotoxin Quantitation kit, following the provided instructions. The absorption at a wavelength of 450 nm was analyzed using an Epoch 2

microplate reader manufactured by Bio-Tek Instruments in Winooski, VT, USA **(Zhuang et al., 2022; Zhao et al., 2023)**.

Assessment of the lipid profile

Allain et al. in 1974 described the enzymatic colorimetric technique for measuring blood total cholesterol (TC). The blood HDL-c was measured using the dextran sulphate-magnesium (II) precipitation technique, as described by **Albers et al. in 1978**. The blood triglyceride levels were determined using the glycerol phosphate oxidase enzymatic technique, as described by **Bucolo and David in 1973**. The VLDL-c was determined by dividing the blood triglyceride level by 5, whereas the serum LDL-c was determined using the Friedewald formula **(Friedewald et al., 1972; Warnick et al., 1990)**.

Assessment of tissue structure

We immersed the splenic tissue specimens in 10% neutral buffered formalin for 48 hours to fix them. Then, they were dehydrated using progressively higher concentrations of ethyl alcohol, cleaned with xylene, embedded in paraffin wax, cut into sections that were 5μm thick, and finally stained with hematoxylin and eosin (H&E) following the protocol described by **Bromma et al. (2022)**.

Assessment using immunohistochemical techniques

We placed paraffin slices on positively charged slides and microwaved antigen retrieval in citrate buffer (pH 6.0) for 10 minutes to identify CD3 and CD68. Afterwards, the natural peroxidase was inhibited with H_2O_2 for a duration of 30 minutes. The slides were treated with mouse monoclonal anti-CD3 and anti-CD68 antibodies at a dilution of 1:250 (catalog numbers sc-20047 and sc-20060, respectively, from Santa Cruz Biotechnology Inc., CA, United States). The treatment was carried out overnight at a temperature of 4 °C. Subsequently, the sections were subjected to treatment with secondary antibodies that were linked to the streptavidin-biotin-peroxidase complex. The chromogen utilized was Diamino-benzidine (DAB). Hematoxylin was used to counter-stain all tissue slices **(Emam et al., 2023)**.

Methods for assessing histological and immunohistochemical analysis

Meyerholz and Beck (2018) utilized the ordinal scoring approach to assess the histological alterations in splenic tissues across several groups. The ratings ranged from 0 to 4, with 0 indicating normal, 1 indicating less than 25% affection, 2 indicating 25% to 50% affection, 3 indicating 50% to 75% affection, and 4 indicating more than 75% affection. Quantitative immunohistochemical analysis of CD_3 and CD_{68} expressions in all examined splenic tissues was conducted using Image J 1.47 software (National Institutes of Health, Bethesda, United States) **(Andrejčáková et al., 2021)**. The analysis involved scoring the brown color intensities as the relative optical density of the DAB reaction. Three slides were examined using the Leica DM3000 microscope. Each slide had five randomly selected pictures, which were analyzed for histology and immunohistochemical grading. The images were seen at a resolution of 400X.

Quantitative analysis

We conducted each experiment three times and repeated it at least three times. The results were reported as the mean value plus or minus the standard deviation (SD). The statistical significance of the data was assessed using one-way ANOVA and t-test. A p-value less than 0.05 was deemed to indicate a statistically significant difference **(Kulshrestha et al., 2020)**.

RESULTS AND DISCUSSION

Hematological analysis refers to the examination and evaluation of blood samples to assess various aspects of blood composition and function

Regarding the total blood count, the BPS-treated group showed notable decreases in Hb concentration, PCV, PLTs, RBCs, MCV, MCH, and MCHC compared to the control group. There was a considerable rise in the count of white blood cells (WBCs). Compared to the BPS group, the metrics showed big improvements in the *Astragalus membranaceus, Glycyrrhiza glabra*, *Iris germanica*, and mixed groups. The herb-treated groups exhibited a notable reduction in the count of white blood cells (WBCs) compared to the positive control group, as seen in Tables 1 and 2. Based on these findings, we may deduce that bisphenol S leads to elevated levels of white blood cells, reduced levels of hemoglobin and platelets, as well as a drop in the number of red blood cells. Conversely, administering *Astragalus membranaceus*, *Glycyrrhiza glabra*, *Iris germanica*, or a combination of these substances counteracted the negative impact of BPS on the erythrogram, leukogram, and other measures.

Table (1): immunomodulatory effects of *Astragalus membranaceus*, *Iris germanica*, *Glycyrrhiza glabra* and its active components on CBC in male CD1 mice

Groups/ Variables	Hb (g/dL)	PCV	WBCs (K/µl)	PLTs (×10 K/µl)
Extract	Mean±SD	Mean±SD	Mean±SD	Mean±SD
-Ve	16.1±1.01^{a}	45.70±1.26^{a}	9.01±0.10^{e}	159.40±5.06^{a}
+Ve	10.0±0.47^{d}	12.43±1.64^{e}	20.08±0.03^{a}	67.12±2.38^{f}
Astragalus membranaceus	13.9±1.13^{c}	27.18±1.39^{d}	16.65±0.27^{b}	86.43±3.17^{e}
Glycyrrhiza glabra	14.2±1.17^{b}	34.25±1.07^{c}	12.83±0.49^{c}	97.18±3.45^{d}
Iris germanica	15.0±0.69^{b}	38.46±2.12^{b}	11.06±0.82^{d}	132.03±4.16^{c}
Mix	16.9±1.23^{a}	46.04±1.39^{a}	9.74±0.05^{e}	146.08±3.09^{b}

- Results are expresses as Mean±SD.
- Values with different letters in each row are significantly different (P≤0.05), while the difference between those with wholly or partly the same letters is not significant.

Table (2): immunomodulatory effects of *Astragalus membranaceus*, *Iris germanica*, *Glycyrrhiza glabra* and its active components on CBC in male CD1 mice

Groups/ Variables	RBCs (M/µl)	MCV (fl)	MCH	MCHC (g/dL)
Extract	Mean±SD	Mean±SD	Mean±SD	Mean±SD
-Ve	10.45±0.10^{b}	95.24±2.06^{a}	35.83±3.17^{a}	36.38±2.65^{a}
+Ve	6.37±0.14^{e}	43.18±1.50^{e}	18.26±2.15^{e}	25.73±2.80^{d}
Astragalus membranaceus	8.04±0.18^{d}	56.25±2.17^{d}	22.95±2.28^{d}	28.54±2.39^{c}
Glycyrrhiza glabra	9.12±0.20^{c}	76.08±3.53^{c}	26.04±2.87^{c}	30.14±3.72^{b}
Iris germanica	9.45±0.23^{c}	84.74±3.10^{b}	29.16±3.29^{b}	32.73±2.22^{b}
Mix	11.13±0.19^{a}	97.24±3.78^{a}	36.35±2.46^{a}	34.58±2.35^{a}

- Results are expresses as Mean±SD.
- Values with different letters in each row are significantly different (P≤0.05), while the difference between those with wholly or partly the same letters is not significant.

Organs responsible for immunity

The results in Table 3 show how *Astragalus membranaceus*, *Iris germanica*, *Glycyrrhiza glabra*, and their active ingredients affected the immune systems of male CD1 mice in the study groups. Rats exhibited a notable modification in both the thymus index and the spleen index. The thymus index and spleen index were considerably lower in the control negative group compared to the control positive group. The average values of both the thymus index and spleen index significantly decreased in all tested groups (G3, G4, G5, and G6) compared to the positive control group. Compared to the other treatments, G6 had the lowest thymus index and spleen index scores. Herbal preparations, particularly tonics, widely utilize *Astragalus membranaceus* to enhance the immune system's resilience. Practitioners administer *Astragalus membranaceus* to adults with compromised immune systems, individuals with chronic conditions such as diabetes and cancer accompanied by mild inflammation, and those experiencing physiological stress **(Wu et al., 2018)**. Herbal preparations of *A. membranaceus* have been shown to change the immune system by affecting the thymus and spleen, lymphatic tissues, the bursa of Fabricius in birds, and dendritic cells in the bone marrow **(Yu et al., 2018)**. The preparations of *A. membranaceus* can enhance the proliferation of primary stem cells in lymph nodes, specifically B and T lymphocytes, and control the activity of natural killer cells and macrophages **(Zheng et al. 2020)**. A group of proteins called nuclear factor-kappa B (NF-κB) transcription factors are very important for turning on the immune system and controlling how immune signals are sent **(Liu et al., 2017; Qader et al., 2020)**. NF-κB is a transcription factor group that controls gene expression in response to immune and inflammatory reactions in the body. This review will specifically examine the immunomodulatory effects of *A. membranaceus*, particularly in relation to NF-κB. It will also explore the chemical ingredients and natural products that are responsible for this action.

Table (3): immunomodulatory effects of *Astragalus membranaceus, Iris germanica*, *Glycyrrhiza glabra* and its active components on immunity organs in male CD1 mice

Groups/ Variables	Thymus index (%)	Spleen index (%)
Extract	Mean±SD	Mean±SD
-Ve	0.075 ± 0.04^{e}	0.049 ± 0.08^{f}
+Ve	0.183 ± 0.01^{a}	0.125 ± 0.09^{a}
Astragalus membranaceus	0.129 ± 0.03^{b}	0.110 ± 0.01^{b}
Glycyrrhiza glabra	0.097 ± 0.09^{c}	0.092 ± 0.02^{c}
Iris germanica	0.081 ± 0.07^{d}	0.073 ± 0.15^{d}
Mix	0.073 ± 0.05^{e}	0.057 ± 0.04^{e}

- Results are expresses as Mean±SD.
- Values with different letters in each row are significantly different ($P\leq0.05$), while the difference between those with wholly or partly the same letters is not significant.

Proinflammatory cytokines

In Table 4, you can see how *Astragalus membranaceus*, *Iris germanica, Glycyrrhiza glabra*, and their active ingredients affected proinflammatory cytokines in male CD1 mice. Noticeable changes were seen in the levels of IL-1β, IL-6, and TNF-α in rats. The levels of IL-1β, IL-6, and TNF-α were considerably elevated in the control negative group compared to the control positive group. All the groups that were tested (G3, G4, G5, and G6) exhibited a notable rise in the average levels of IL-1β, IL-6, and TNF-α when compared to the positive control group. G6 demonstrated significantly elevated levels of IL-1β, IL-6, and TNF-α in comparison to the other treatments. The negative control group exhibited a substantial reduction in the IL-12 p70 markers as compared to the positive control group. Each treatment group had a substantial

reduction in comparison to the positive control group. The outcomes achieved by Group 6 were superior. Biological or pharmacological stimuli cause immunomodulatory activity, which is the overall impact on immune response components related to bodily fluids or cells. T-lymphocytes are key components of the immune system and consist of T-helper cells (CD^{4+}) and cytotoxic cells (CD^{8+}). T-helper cells exert their influence through the secretion of soluble cytokines and/or direct contact with other cells. Cytotoxic cells eliminate pathogen-infected host cells **(Nazir, 2013)**. Based on the cytokines they produce, we classify CD^{4+} cells as Th type 1 and Th type 2 cells. In order to get rid of intracellular infections, CD^{4+} Th1 cells must be activated. These cells release IL-2 and IFN-γTh2 cells produce IL-4, IL-5, and other substances that regulate the immune response to external organisms, suppress cell-mediated inflammatory responses, and potentially protect against arthritis. An imbalance in the Th1/Th2 ratio towards Th1 cells can lead to immunopathology and the development of autoimmune disorders that affect particular organs. Th2 cells make cytokines that play a big part in atropy and allergic inflammation by turning on mast cells and esinophils and raising IgE levels **(Kaur and Ghorai, 2022)**. We evaluated the immunomodulatory activities of the hydroalcohol extract of *G. glabra* roots on a Naval Medical Research Institute (NMRI) mouse. The study revealed a considerable rise in antibody levels, indicating an improvement in the immune system **(Abtahi Froushani et al., 2014)**. However, a research study found no significant immune-stimulating effect ($P > 0.05$) when administering liquorice extract with drinking water. **Moradi et al. 2013** determined this by testing several immunological parameters related to influenza and Newcastle disease. According to **Guo et al. (2015)**, another study suggests that one of the main ways licorice fights autoimmune and inflammatory diseases is by increasing the production of regulatory T cell examining the effects of low-molecular-weight polysaccharides derived from *G. glabra* on anticancer activity revealed an up-regulation of IL-7. This up-regulation was responsible for the proliferation and maturation of immune cells **(Ayeka et al., 2016)**. Furthermore, the study by **Elabd et al. (2016)** provided additional evidence supporting the use of a diet containing licorice extract for promoting development and enhancing performance. The **2017** study by **Ayeka et al.** also found that polys

accharides from *G. glabra* lowered the levels of TNFα and raised the levels of IL-2, IL-6, IL-7, and serum antitumor cytokines. A new study by **Ng et al. (2021)** showed that the polysaccharides in the ethanol extract of *G. glabra* boost the immune system by raising the levels of IgG, IgM, and IgA in the blood. It also increases the presence of cells in the spleen. Investigations conducted on L. major-infected male CD1 mice demonstrated the considerable therapeutic and immunomodulatory effects of the hydroalcohol extract of *G. glabra* and glycyrrhizic acid **(Sheikhi et al., 2022)**.

Table (4): immunomodulatory effects of *Astragalus membranaceus*, *Iris germanica*, *Glycyrrhiza glabra* and its active components on proinflammatory cytokines in male CD1 mice

Groups/ Variables	IL-1β (pg/ml)	IL-6 (pg/ml)	IL-12 p70 (pg/ml)	TNF-α (pg/ml)
Extract	Mean±SD	Mean±SD	Mean±SD	Mean±SD
-Ve	28.03±5.21[a]	37.52±4.04[a]	3.42±1.13[e]	93.82±5.03[a]
+Ve	5.60±1.47[f]	7.16±2.08[f]	23.40±2.25[a]	24.15±3.24[f]
Astragalus membranaceus	10.23±1.58[e]	15.70±2.45[e]	18.62±2.03[b]	48.09±2.15[e]
Glycyrrhiza glabra	15.70±2.04[d]	19.63±2.51[d]	12.57±0.78[c]	59.08±2.06[d]
Iris germanica	18.34±2.63[c]	25.10±2.48[c]	9.63±1.04[d]	73.40±3.18[c]
Mix	25.23±2.75[b]	32.47±3.17[b]	4.98±0.53[e]	86.06±1.29[b]

- Results are expresses as Mean±SD.
- Values with different letters in each row are significantly different (P≤0.05), while the difference between those with wholly or partly the same letters is not significant.

Immunoglobulins

There were a lot more immunomodulatory effects of *Astragalus membranaceus*, *Iris germanica*, *Glycyrrhiza glabra*, and their active ingredients on immunoglobulins (IgA, IgE, and IgG) in the negative control group of male CD1 mice than in the positive control group (Table 5). All indicators within the remedy categories have shown a substantial rise, with statistical significance at a significance level of P<0.05. G6 showed the most optimal outcomes for IgA, IgE, and IgG. Simultaneously, the IgM test results exhibited a substantial reduction in the negative control group as compared to the positive control group. All treatment groups exhibited a substantial reduction in comparison to the positive control group. Many published studies have extensively evaluated *Glycyrrhiza glabra* as a very cost-effective and readily accessible immunomodulatory agent **(Kumar and Kumar, 2013; Tiwari et al., 2018)**. In addition, a different study found that pure polysaccharides from *G. glabra* could control how macrophages fight off infection **(Cheng et al. 2008)**. On top of that, the water extract of *G. glabra* roots boosted the immune system by raising the phagocytosis test, the hemagglutination antibody titer value, and the delayed type hypersensitivity. **Bagherwal et al.** reported in **2009** that they detected this effect at dosage levels of 150 and 300 mg/kg of body weight. Additionally, some studies show that polysaccharides from *G. glabra* boost the immune system by raising the amounts of IgG, IgM, and IgA in mice's blood serum **(Hong et al., 2009)**. It was also found that giving liquorice increased the production of saliva IgA, which shows that it had immunostimulant effects **(Katayama et al., 2011)**. Researchers showed that glycyrrhizin induced an increase in the expression of CD40, CD86, and MHC-II, primarily responsible for the maturation and function of dendritic cells in the spleen of mice. **Bordbar et al.'s 2012** research supported this by observing greater IL-12 production. Additionally, when combined with zinc, the aqueous extract of *G. glabra* root demonstrated immunomodulatory effects at a dosage of 1.5 g/kg/body weight. **Mazumder et al.** conducted both *in vitro* and *in vivo* investigations in **2012** to establish these effects.

Table (5): immunomodulatory effects of *Astragalus membranaceus, Iris germanica, Glycyrrhiza glabra* and its active components on immunoglobulins in male CD1 mice

Groups/ Variables	IgA	IgE	IgG	IgM
Extract	Mean±SD	Mean±SD	Mean±SD	Mean±SD
-Ve	118.01±3.23[a]	112.00±2.71[a]	109.00±3.42[a]	22.46±2.08[d]
+Ve	80.26±1.76[d]	69.40±2.48[c]	44.62±2.03[d]	60.38±4.25[a]
Astragalus membranaceus	96.40±2.15[c]	94.35±2.41[b]	78.48±3.90[c]	40.52±3.73[b]
Glycyrrhiza glabra	103.23±1.37[b]	97.28±1.03[b]	80.61±2.95[b]	33.60±3.04[c]
Iris germanica	100.00±3.52[b]	97.86±4,71[b]	83.63±2.46[b]	30.49±2.68[c]
Mix	116.00±4.86[a]	113.05±3.43[a]	111.96±3.70[a]	25.91±2.23[d]

- Results are expresses as Mean±SD.
- Values with different letters in each row are significantly different (P≤0.05), while the difference between those with wholly or partly the same letters is not significant.

T-lymphocytes

Table 6 presents the immunomodulatory effects of *Astragalus membranaceus*, *Iris germanica*, *Glycyrrhiza glabra*, and their active components on T lymphocytes in male CD1 mice. The negative control group had a substantial rise in CD^{3+}, CD^{4+}, and CD^{8+} compared to the positive control group. Simultaneously, all treatment groups exhibited a substantial rise in comparison to the positive control group. The G6's outcomes were superior. Research has demonstrated that the compounds glycyrrhizin and glycyrrhetinic acid stimulate the production of interferon. When interferons are activated, they bind to cell surfaces and cause the production of intracellular proteins that stop the transcription of viral DNA. This has strong antiviral effects. After interferon induction, macrophages are activated, and the activity of natural killer cells increases **(Murray, 2020)**. Glycyrrhizin directly stopped the growth of many DNA and RNA viruses in cell cultures. These viruses included HIV, vaccinia, Epstein-Barr, herpes simplex, Newcastle disease, vesicular stomatitis viruses, and the coronavirus linked to severe acute respiratory syndrome (SARS). Additionally, it irreversibly inactivated herpes simplex virus 1 (HSV-1). Administering glycyrrhizin to mice with herpetic encephalitis resulted in a 2.5-fold improvement in their survival rate, while reducing HSV-1 multiplication in the brain to 45.6% of the control group. As previously mentioned, glycyrrhizin also hindered the thymolytic and immune-suppressive effects of cortisone. Additional constituents of licorice also demonstrated immunomodulatory effects **(Zuo et al., 2023)**. *Iris germanica* is a highly abundant reservoir of isoflavones. Irisolidone and irilone, two isoflavones derived from this plant, function as immunomodulators. In the male CD1 mouse model, their influence on the generation of T lymphocyte cells (specifically CD^{4+} and CD^{8+}) and cytokines (including IFN-γ, IL-2, IL-4, and IL-5) was documented. Oral administration of Irilone reduces the levels of these cytokines, thereby exhibiting immunosuppressive properties. As a result, it may be beneficial in situations such as organ transplantation. In pathological circumstances characterized by changes in T lymphocyte equilibrium **(Yousefsani et al., 2021)**, the use of irisolidone can provide advantages. This study examines the immunomodulatory effects of two isoflavones, specifically 5,7-dihydroxy-6,4′-dimethoxyisoflavone (irisolidone) (1) and 5,4′-dihydroxy-6,7-methylenedioxy-isoflavone (irilone) (2), extracted from *Iris germanica* (*Iridaceae*). We looked at how they affected the production of T-lymphocytes (mainly CD^{4+} and CD^{8+} cells) and T-cell cytokines, such as Th1: IL-2, IFN-γ, and Th2: IL-4 and IL-5, in male CD1 mice using a flow cytometric method. The study found that their influence varied depending on the dosage. Administering medicines orally at dosages ranging from 0.1 to 0.8 mg/kg per dose has shown that drug 1 has a stimulating effect on T-cells and the generation of Th1 cytokines, whereas drug 2 serves as an

immunosuppressant for both cells and cytokines. Compounds 1 and 2's methylation derivatives showed a similar pattern to their original compounds, but their effectiveness significantly decreased, underscoring the importance of unbound phenolic groups for their immunomodulatory properties **(Nazir et al., 2009)**.

Table (6): immunomodulatory effects of *Astragalus membranaceus, Iris germanica*, *Glycyrrhiza glabra* and its active components on T-lymphocytes in male CD1 mice

Groups/ Variables	$CD3^+$	$CD4^+$	$CD8^+$
Extract	Mean±SD	Mean±SD	Mean±SD
-Ve	597.68 ± 2.08^a	800.03 ± 2.42^b	843.29 ± 2.74^a
+Ve	12.38 ± 1.26^f	245.84 ± 1.09^f	150.07 ± 1.14^e
Astragalus membranaceus	145.06 ± 2.18^e	375.32 ± 2.46^e	306.27 ± 2.23^d
Glycyrrhiza glabra	253.38 ± 3.25^d	493.84 ± 2.57^d	463.51 ± 1.60^c
Iris germanica	328.63 ± 2.30^c	673.21 ± 2.04^c	641.28 ± 2.48^b
Mix	519.03 ± 2.29^b	835.49 ± 2.94^a	865.07 ± 2.48^a

- Results are expresses as Mean±SD.
- Values with different letters in each row are significantly different (P≤0.05), while the difference between those with wholly or partly the same letters is not significant.

Analysis of lipids

If you look at Table 7, you can see how *Astragalus membranaceus*, *Iris germanica*, *Glycyrrhiza glabra*, and their active ingredients changed the lipid profile in male CD1 mice, which helps the immune system. The negative control group had a substantial reduction in TC, TG, LDL-c, and VLDL-c levels as compared to the positive control group. There was a substantial reduction in all treatment groups as compared to the positive control group. There was a substantial rise in HDL-c levels in the negative control group compared to the positive control group. Additionally, all treatment groups showed a significant increase in HDL-c levels compared to the positive control group. The findings indicated that the sixth group had superior performance.\

Table (7): immunomodulatory effects of *Astragalus membranaceus, Iris germanica*, *Glycyrrhiza glabra* and its active components on lipids profile in male CD1 mice

Groups/ Variables	TC	TG	HDL-c	LDL-c	VLDL-c
Extract	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
-Ve	143.53 ± 1.10^f	90.08 ± 0.85^f	65.53 ± 1.35^a	59.8 ± 0.93^f	18.02 ± 0.17^f
+Ve	385.13 ± 2.32^a	328.43 ± 3.57^a	38.46 ± 1.21^e	280.98 ± 3.85^a	65.69 ± 0.41^a
Astragalus membranaceus	275.04 ± 2.19^b	286.21 ± 2.75^b	40.79 ± 2.85^d	177.01 ± 2.74^b	57.24 ± 0.56^b
Glycyrrhiza glabra	234.1 ± 1.82^c	245.4 ± 2.91^c	46.32 ± 2.67^c	138.7 ± 1.63^c	49.08 ± 0.38^c
Iris germanica	176.8 ± 1.23^d	198.5 ± 1.68^d	51.25 ± 0.19^b	85.61 ± 0.57^d	39.7 ± 0.25^d
Mix	155.3 ± 1.17^e	145.8 ± 1.32^e	62.73 ± 1.24^a	63.43 ± 0.58^e	29.16 ± 0.14^e

- Results are expresses as Mean±SD.
- Values with different letters in each row are significantly different (P≤0.05), while the difference between those with wholly or partly the same letters is not significant.

Exposure to BPS causes damage and inflammation in the spleen

We exposed the mice to either BPS or olive oil (as a control) for 55 consecutive days. We employed a spleen coefficient to assess the level of exposure to BPS. The results showed a significant difference in spleen coefficient between the group treated with BPS and the control group (P<0.01). Significantly, we observed a significant rise in the spleen coefficient in the group treated with BPS compared to the control group. The spleen may be particularly

susceptible to demonstrating the harmful effects of BPS compared to other organs **(Azevedo et al., 2020)**.

The pathophysiological processes underlying splenic damage caused by BPS exposure are described

The spleen is a vital component of the immune system, responsible for producing both innate and adaptive immune responses. Notably, the spleen serves as the primary blood filter that separates the bloodstream from the organs. Our study showed that giving BPS to animals caused a lot of damage to the spleen. The spleen got bigger, had higher levels of pro-inflammatory cytokines (IL-6), fewer cells in the periarteriolar lymphoid sheath and primary lymphoid follicles with low cellularity, and different amounts of So, SM, and Cer in different places at the molecular and histopathological levels **(Li et al., 2023)**. Complex lipid signaling has the ability to regulate the spleen's immunological responses, including both immune responses and trafficking. In comparison to the control group, exposure to BPS resulted in an increase in the amount of sphingosine-1-phosphate (S1P). This molecule plays a vital role in transmitting lipid signals from the immune responses in the spleen. The lipidome study and gene expression confirmed that Cer, SM, and So are significant contributors to the variance of S1P. The results of our study showed that BPS exposure raised the amounts of So and CDase while lowering the amounts of SM and Cer and the expression of SMase. These changes might potentially account for the difference in S1P. Significantly, exposure to BPS can trigger immunological responses mediated by lipids, both in the innate and adaptive immune systems, through binding to S1P receptors, resulting in increased levels of S1P. Exposure to BPS **(Martínez, 2021)** has the potential to cause splenic damage.

Spleen histopathology

Figure 1 depicts a typical spleen, which consists of a clearly defined red pulp responsible for blood filtration and a white pulp containing discrete lymphoid compartments for immunological function. The marginal zone divides these two regions. A protective covering known as a capsule and thin strands known as trabeculae uphold the structure of the spleen. The spleen tissue that was affected by BPS (100 μg/kg body weight/day) is shown in Figure 2. This tissue shows major pathological changes. The spleen experienced a significant increase in size. Upon careful observation, the outside layer of the structure displayed abnormal swelling, while the inner tissue appeared uniformly congested with scattered regions of bleeding. The white pulp showed significant atrophy, marked by the near-total absence of the typical lymphoid follicles and per arteriolar lymphoid sheaths. The red pulp exhibited significant enlargement and congestion, characterized by a multitude of dilated sinuses filled with blood. The red pulp showed an abundant infiltration of lymphocytes, plasma cells, and macrophages. The clear boundary between the red and white pulps was not visible because of the intense inflammation and congestion. We lost the marginal zone, which typically acts as a boundary between the white and red pulp compartments. The capsule and trabeculae showed an enhanced accumulation of collagen fibers. The histological analysis of G1 (which was given 200 mg/kg of *Astragalus membranaceus* extract every day) and G2 (which was given 200 mg/kg of *Glycyrrhiza glabra* extract every day), as shown in Figures 3, showed that the white pulp had some lymphoid follicles and periarteriolar lymphoid sheaths restored (see pictures A and B). There was a reduction in red pulp congestion and macrophage infiltration. The boundary between the red and white pulp divisions became more clearly defined. The marginal zone began to regenerate, creating a division between the white and red pulp. Collagen fiber deposition in the capsule and

trabeculae decreased. As shown in photo A, Figure 4 shows that the splenic tissue improved significantly in G3 (200 mg/kg/day of *Iris germanica* extract), with less inflammation and some restoration of the normal structure of the spleen. We administered G4 at a dosage of 200 mg/kg/day, a mixture of herbal extracts in a 1:1:1 volume-to-volume ratio. After looking at Photo B, it is clear that the splenic tissue has improved significantly. There is less inflammation, less necrosis, and more changes that are related to regeneration.

Figures and Tables

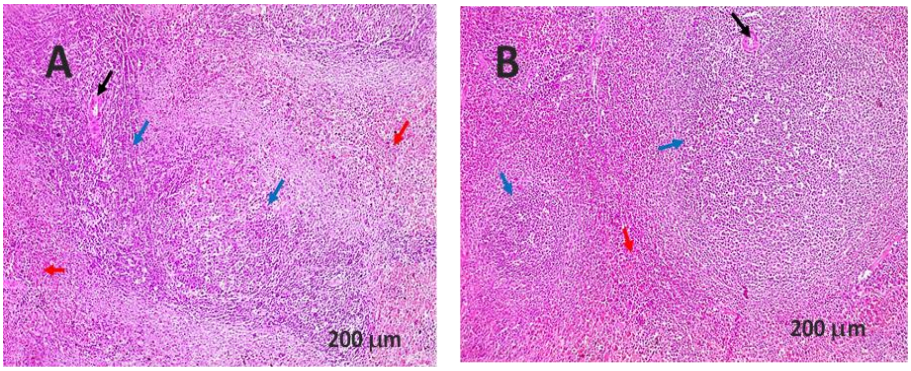
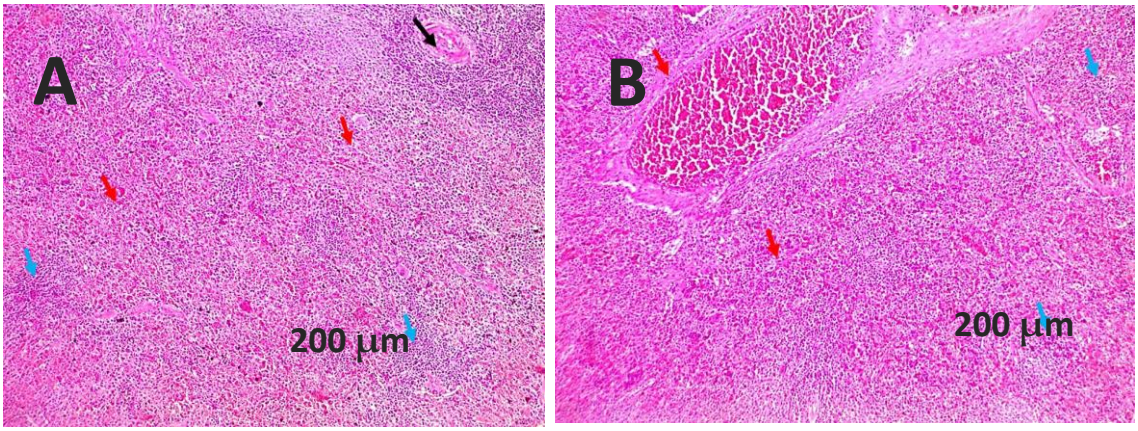
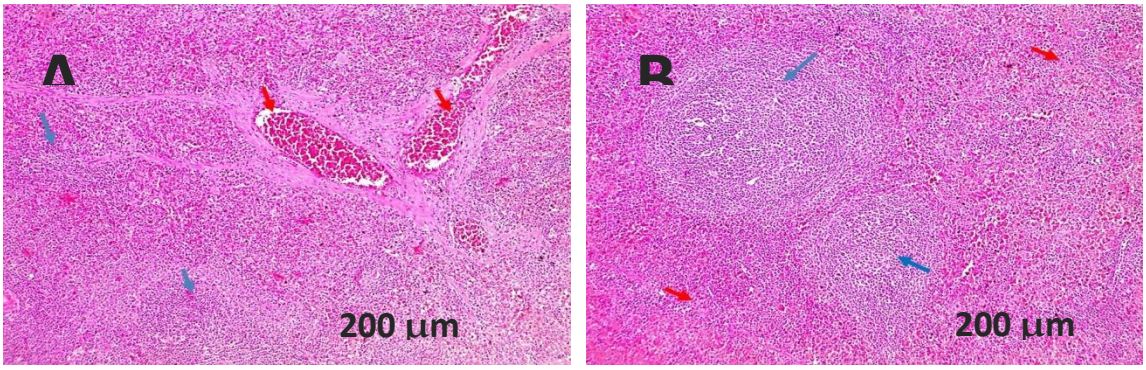


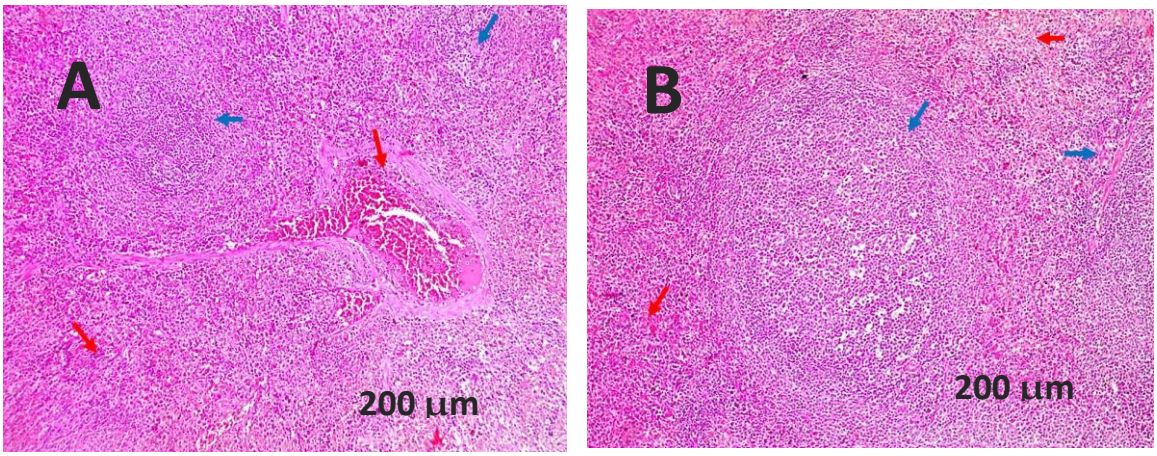
Figure 1 (A, B) The normal spleen (control) slice had white pulp of normal size, consisting of lymphoid follicles shown by blue arrows. A black arrow indicated the presence of a central arteriole within the white pulp. The red pulp, including blood sinusoids, appeared to be of average size, as indicated by the red arrows. We used H&E X 200 magnification for this observation.



The inebriated spleen (+Ve) in Figure 2 (A, B) exhibits significant congestion. The red pulp appears dilated and congested, with additional medullary hematopoiesis indicated by the red arrows. The white pulp around it appears atrophic, as shown by the blue arrows. The black arrow also indicates the presence of a hyalinized central arteriole. We used H&E staining at a magnification of 200X to make these observations.



There was a lot of red pulp congestion (shown by red arrows) and a small increase in the size of the white pulp (shown by blue arrows) in sections [3 (A, B)] of the spleen from groups G1 (which was given 200 mg/kg/day of Astragalus membranaceus) and G2 (which was given 200 mg/kg/day of Glycyrrhiza glabra). The H&E staining at 200X showed these changes.



In Figure 4 (A, B), the spleen segment from G3 (fed 200 mg/kg/day of Iris germanica) and G4 (fed a 200 mg/kg/day mix of herbs) showed a lot of red pulp congestion (shown by red arrows) and a small increase in the size of the white pulp (shown by blue arrows) when stained with H&E at a 200X magnification. Photo B clearly shows a substantial and more noticeable enhancement in the splenic tissue, together with a continued decrease in inflammation, less necrosis, and more widespread regeneration changes

CONCLUSION

This study demonstrated the histological effects of BPS poisoning on the spleens of mice. The obtained data showed a significant toxicity in the spleen, characterized by splenomegaly and morphological changes in the groups treated with BPS. Exposure to BPS may lead to splenomegaly due to the heightened production of pro-inflammatory cytokines, a significant reduction in immune cells, and an increase in the levels and distribution of lipid markers in immune cells inside the white pulp. The findings paved the way for exploring the novel mechanisms linking lipid regulation to inflammatory responses in bisphenol S-induced splenic toxicity. We now know more about lipid metabolism in the field of environmental toxicology. This also helps us understand how *Astragalus membranaceus*, *Glycyrrhiza glabra*, *Iris germanica*, and their active ingredients might affect mice that have been exposed to BPS.

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