Journal of Futuristic Sciences and Applications
Vol. 7(2), Jul-Dec 2024, pp. 6-14
doi: 10.51976/jfsa.722402
www.gla.ac.in/journals/jfsa
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Immunomodulatory and Toxicological Assessment of a Synergistic AYUSH-Based Herbal Formulation Against ParaTB Infection

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ABSTRACT

The increasing prevalence of Mycobacterium avium subspecies paratuberculosis (MAP) infection and limitations associated with conventional anti-tubercular drugs necessitate exploration of safe alternative therapeutics. The present study evaluated the immunomodulatory and toxicological potential of a synergistic AYUSH-based formulation (SABF) prepared from Ocimum sanctum and Solanum xanthocarpum. The formulation was assessed through splenocyte proliferation assay, cytokine profiling, antioxidant studies, and acute oral toxicity evaluation in Wistar rats. Significant enhancement in splenocyte proliferation and modulation of cytokines including IL-2, IFN-γ, TNF-α, IL-4, IL-6, and IL-10 were observed. Antioxidant analysis revealed strong free radical scavenging activity. Toxicity studies demonstrated no significant alteration in hematological, biochemical, or histopathological parameters even at 2000 mg/kg dose. The study concludes that SABF possesses potent immunomodulatory activity with excellent safety profile and may serve as a potential herbal therapeutic against paraTB infection.

Keywords: *Immunomodulation; AYUSH; Toxicity; Cytokines; Splenocytes; ParaTB.*

1.0 Introduction

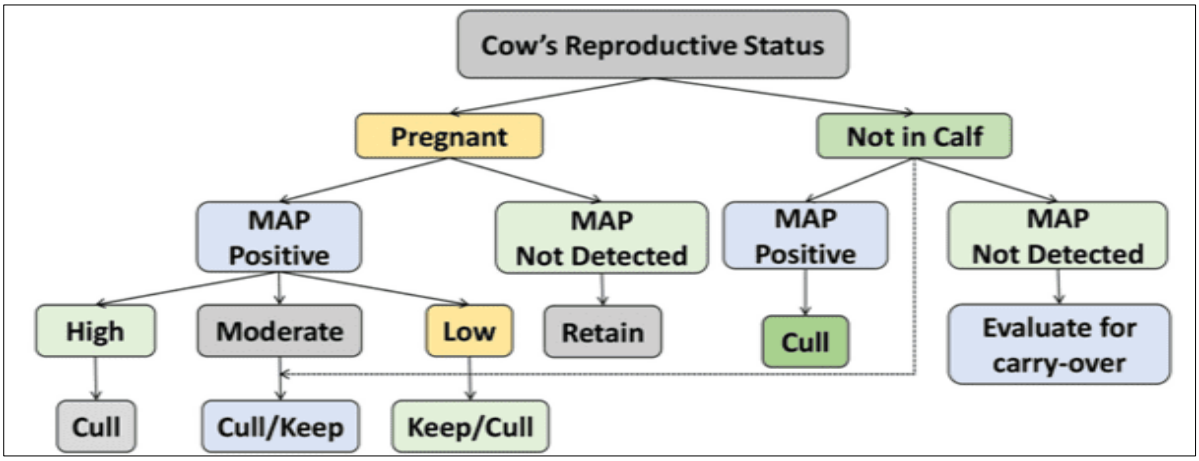
Paratuberculosis (Johne's disease) is caused by Mycobacterium avium subspecies paratuberculosis (MAP), highly resistant, extremely fastidious, Gram-positive acid-fast bacilli. The disease is characterized by reduced productivity, loss in body weight, and can be experienced with (in cattle and buffaloes) or without (in goats and sheep) diarrhea. Animals acquire infection in the early stages (prenatal) through semen or in utero, with colostrum and milk, or from the environment after birth via the fecal–oral route. The high endemicity of MAP in domestic livestock leads to high propagation in herds and flocks. As it is highly resistant in a few bacilli, MAP requires multidrug therapy for a long time (7–8 months), which is not cost-effective even when the cost of animals is not considered. Johne's disease in ruminants is caused by Mycobacterium avium subsp. paratuberculosis (MAP), a persistent bacterium with significant economic effects and global dissemination [1].

The apparent correlation between Mycobacterium avium subspecies paratuberculosis and Crohn's disease in individuals is still being studied extensively, with conflicting results [2]. In 1895, German researchers Johne and Frothingham acknowledged MAP for the first time [3]. As shown in (Figure 1), it commonly infects ruminants (cattle, sheep, goats, deer, etc.), but it has also been reported in non-ruminants, notably wildlife [4].

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Annual cattle sector losses in the United States are estimated to be between $250 million [5] and $1.5 billion.

Figure 1: Control of Mycobacterium Avium Subspecies Paratuberculosis (MAP) Infection in Domestic Livestock



According to a new assessment of available data, which employed a Bayesian technique calibrated for susceptivity and explicitness, the underlying frequency of MAP in dairy cattle in the United States was 91.1%, not the 70.4% claimed in 2007 [6]. The incidence of MAP in beef cattle herds is 7.9% [7]. Even though JD was initially discovered in the United States during the early 1900s, the emphasis on investigation and disease prevention alone has expanded in the last 20 years. To combat Johne's disease on a farm and recognize herds with minimal infection susceptibility, a discretionary Bovine JD Management Program is in operation.

The examination of ambient stool specimens via culturing through elevated sites is among the most cost-effective and highly reliable diagnostic techniques for JD [6] Ironically, wildlife repositories may disrupt initiatives to reduce Johne's disease in livestock unless their significance in wildlife is completely defined [8]. JD transmission is reduced when improved diagnoses are combined with good management strategies [9].

2.0 Defense Mechanism of Host through Alveolar Macrophages

The elimination of the tuberculosis infection is significantly aided by alveolar macrophages. TB transmits from one host to the other in the form of droplets while sneezing and coughing. The polysaccharides and lipids present on the cell wall of Mycobacteria act as antigens and stimulate the immune cells by first interfering with the macrophages. The macrophages are recruited to the blood stream and maintain the infection in the host. The presence of cholesterol in the macrophage is essential for engulfing the bacteria into the macrophages. The cellular receptors identify the antigens of the bacilli and internalize them. Macrophages therefore stimulate the production of pro-inflammatory cytokines and the migration of immune cells to the site of infection [10].

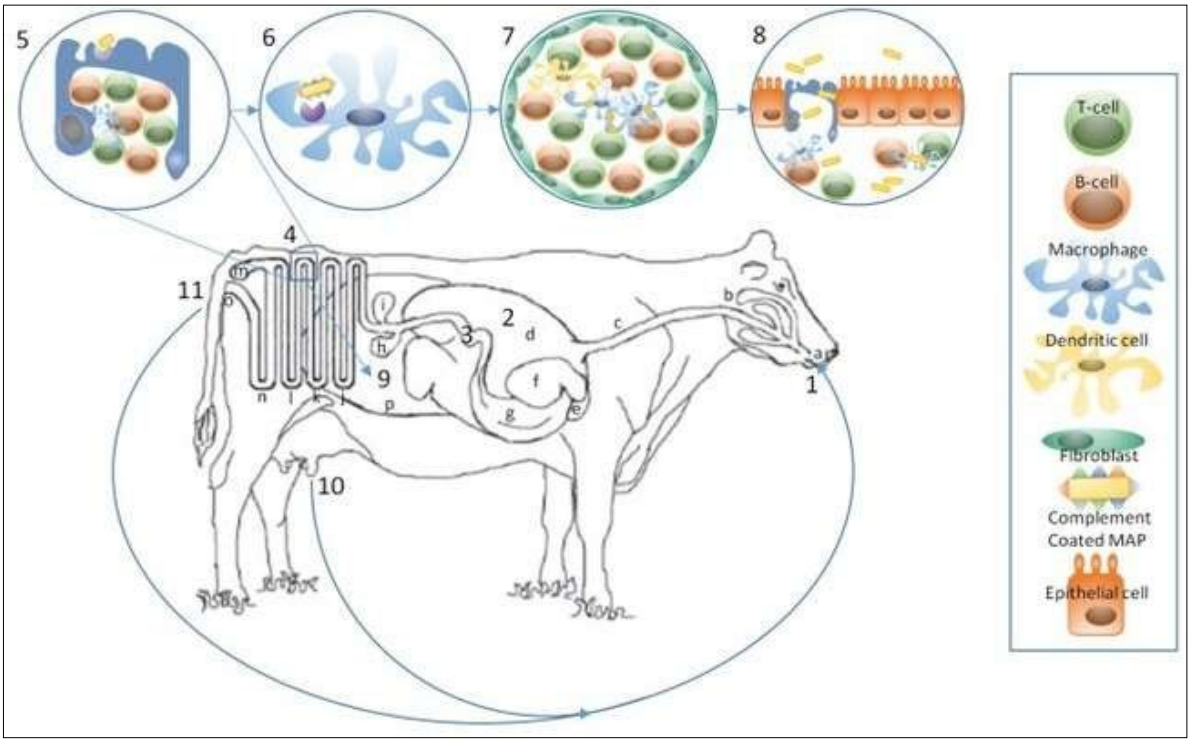
Macrophages stimulate the activation of T cells and engulf the bacilli [11]. Macrophages are made up of virulent mycobacteria, as well as epithelial cells and monocytes that induce or migrate uninfected macrophages, allowing them to phagocytoze infected apoptotic cells. If the macrophages

Doi: 10.51976/jfsa.722410

fail to show microbicidal activity, the bacterial burden is increased within the macrophages. During this stage, mycobacteria disseminates at an early stage. M.tb infects and resides in phagosomes.

It arrests the fusion of phagosome and lysosome so that the maturation of the phagosome to phagolysosome is arrested. It has been reported that M.tbis able to disrupt its phagosome and to translocate into the host cell cytoplasm [12]. During the treatment of M.tb infected animals with morpholino, the abnormal vasculature was decreased, demonstrating that macrophages mediate new vessel growth as a factor causing inflammation. After 2 days, M.tb progressively translocates from phagolysosomes into the cytosol in non-apoptotic cells [13]. Depletion of cholesterol levels diminishes the viability of M.tbbacilli. Few studies have found statins to have a pleiotropic effect in terms of tissue damage reduction and immune-modulation.Mycobacteria use the cholesterol found in their hosts' macrophages [14].

Figure 2: M.paratuberculosis Prevalence and Control



The trade of sick animals is typically linked to the spread of M. paratuberculosis. The exact infection prevalence and monetary costs of Johne's illness are unknown because there are no feasible diagnostic tools that can accurately identify M. paratuberculosis subclinical infections. To ensure that herds are unlikely to be infected, national surveillance programmes have been developed in Australia and planned in Canada and the US [15]. The stated prevalence of Johne's illness in each nation, however, is a reflection of the volume and rigour of testing because there isn't a quick, accurate diagnostic assay. Effective surveillance programmes require ongoing testing and monitoring. Australia was the first country to find Johne's disease in cattle and sheep, 75 and 24 years respectively. There were officially 442 sheep flocks and 1968 cow herds with M. paratuberculosis infections in 1998, respectively [16]. According to (Boelaert *et al.*, 2000), the reported sero-prevalence of M. paratuberculosis infection in Belgian dairy cattle is 18%. In Austria, sero-prevalence

of sero-positive cattle of all ages ranged from 0.5 to 7.1 percent, depending on the ELISA method utilized [17]. Sero-prevalence of M. paratuberculosis infection in cow populations have been identified in several investigations conducted in various regions of the United States of America. Sero-prevalence of M. paratuberculosis infection in cow populations have been identified in several investigations conducted in various regions of the USA, ranging from 1.6 to 55 percent [18,19]. Based on histological lesions, the incidence of M. paratuberculosis infections was found in randomly chosen Canadian slaughterhouses. An rise in the number of vulnerable hosts and environmental degradation, which produces soil and water acidity, are further variables impacting the ecology of M. paratuberculosis infections [20].

Animals may now be more vulnerable to Mycobacterium after the eradication of Mycobacterium tuberculosis and Mycobacterium bovis from human and animal populations. Given that M. paratuberculosis is far more resistant to being inactivated by low pH than other pH-resistant bacteria like Yersinia enterocolitica and Listeria monocytogenes, environmental pollution may have given rise to a niche for this disease [21].

3.0 Diagnosis of M.paratuberuclosis (Mycobacterium Avium Subspecies Paratuberculosis (MAP))

MAP is emitted in high concentrations in the faeces and in lesser concentrations in the colostrum and milk of diseased animals. It can stay alive on pasture for more than a year and is resistant to environmental conditions; survival in water outlasts that in soil. The illness is often acquired orally and fecally; it is unknown at what dose an animal can get infected. By introducing sub-clinically infected carriers, herds or flocks contract MAP [22]. Early in life, frequently shortly after birth, animals can contract an infection, although clinical symptoms rarely appear until the animal is sexually mature. Clinical illness progresses gradually. Age causes a rise in infection resistance, but it never reaches 100%. An animal can become infected by whipping and preparing themselves in a pollutedatmosphere, drinking milk, solid food, or water that has been contaminated with MAP, or ingesting the animal while breast feeding on pollutednipples. Intrauterine infections are seen in the later, bacteremic phases of illness [23].

Macrophages present in the gastrointestinal tract are infected by the pathogen living inside the cells and after ingestion also affects related lymph nodes and absorbed in the lower small intestine specifically inside its Peyer's patches. It is conceivable for some animals to get rid of an infection via immunological response, although it is unknown how frequently this happens [24]. The majority of the time, the individuals grow thenat the end cause a longterm disease names as granulomatous enteritis that obstructs nutritional absorption which results in the sickness typical of more severe illnesses. This can taketime of years or can be in some months to manifest and is typically accompanied by an increase in serum antibodies, a reduction in cell-mediated immunity, and blood poisoning with the spread of the infection outside of the gastrointestinal tract. Organisms in this "silent" phase of illness are origin of transmit the diseases because feces start to shed before clinical symptoms become visible [25,26].

4.0 Results and Discussion

The SABF exhibited strong antioxidant activity with significant DPPH radical scavenging potential. Splenocyte proliferation studies showed dose-dependent enhancement of immune cell

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activity compared to control groups. Cytokine profiling revealed increased expression of IL-2 and IFN-γ, indicating stimulation of Th1 immune response. TNF-α levels were significantly reduced, demonstrating anti-inflammatory action. Balanced regulation of IL-4, IL-6, and IL-10 suggested effective modulation of both pro-inflammatory and anti-inflammatory pathways.

Toxicity evaluation demonstrated no mortality or behavioral abnormalities in any treatment group. No significant differences were observed in body weight, organ-to-body weight ratio, liver function tests, renal function parameters, or lipid profile compared to controls.

4.1 Distribution

The Holy basil plant is widely distributed throughout India, and researchers from the Central University of Punjab and Bathinda have studied the large-scale phylogeny graphical of this species using chloroplast whole genomic sequencing, which revealed that this holy basil plant originated from North-Central India [27].

4.1.1 Medicinal uses of bioactive compounds

Ocimum sanctum is a native herb in India. Also known as 'Tulsi,' it belongs to the Lamiaceae family. The Hindu religious tradition is sacred, and Tulsi is perhaps viewed as the most significant plant used in Ayurvedic medicine [28]. Tulsi plants grow in abundance around Hindu temples and can be found in multiple varieties, e.g., green, red, or with a strong but pleasant aroma. In the previous decade, several shreds of scientific evidence have reported (Mandal *et al.*, 1994; Sembulingam *et al.*, 1997; Banu *et al.*, 2009) that holy basil has been utilized to treat a variety of many critical diseases (Godhwani *et al.*, 1987), including asthma, arthritis, heart problems, eye disorders, blood glucose levels, hepato protective, anticancer, anti-fungal, antimicrobial, chronic fever, anti-fertility, and bronchitis [29,30].

Table 1: Biological Mechanism between Bioactive Constituents with MIC50 Values

S. No.	Bioactive Constituents	MIC_{50} Value	Mechanism	References
1	Eugenol	500 µg/ml	Antifungal	Ahmad *et al.*, 2015
2	Linalool	0.12%	Antimicrobial	Federman *et al.*, 2016
3	Ursolic acid	12 µg/mL	Anti-MAP	Navabharat M *et al.*, 2022
4	beta-caryophyllene	32 µg/mL 1024µg/ml	Antimicrobial	Santos *et al.*, 2021
5	Propionic acid	0.25% 0.125%	Antimicrobial Antifungal	Haque *et al.*, 2009
6	Rosmarinic acid	1.2 mg/mL 0.3 mg/mL 2.5 mg/ml	Antimicrobial Antifungal Antiviral	Abedini *et al.*, 2013
7	Apigenin	>4 mg/ml	Antimicrobial	Nayaka *et al.*, 2014
8	Orientin	500 µg/mL 1000 µg/ml	Antimicrobial	Karpiński *et al.*, 2019
9	Isothymusin	200 µg/mL	Antimicrobial	https://www.chemfaces.com/natural/Isothymusin-CFN97562.html accessed on 5 July 2022
10	Vicenin-2	>188µg/mL	Antimicrobial Antifungal	Mohotti *et al.*, 2020

Ocimum sanctum has many chemical constituents, such as carvacrol, eugenol, limatrol, linalool, ursolic acid, caryophyllene, propionic acid, methyl carvicol, Rosmarinic acid, Apigenin, cirsimaritin, Orientin, isothymusin, and Vicenin. Supplementary materials Figures 3.1 and 3.2 show the biological mechanism between the a fore mentioned bioactive constituents and their MIC50 values are given in Table 3.1. Previous research has also showed that the leaf juice extracted from Tulsi demonstrates the inhibition of complete growth in anti-viral and anti-mycobacterial activities [31].

Figure 3: Structures and IUPAC Names of 10–12 Bio Molecules of Ocimum Sanctum Plant

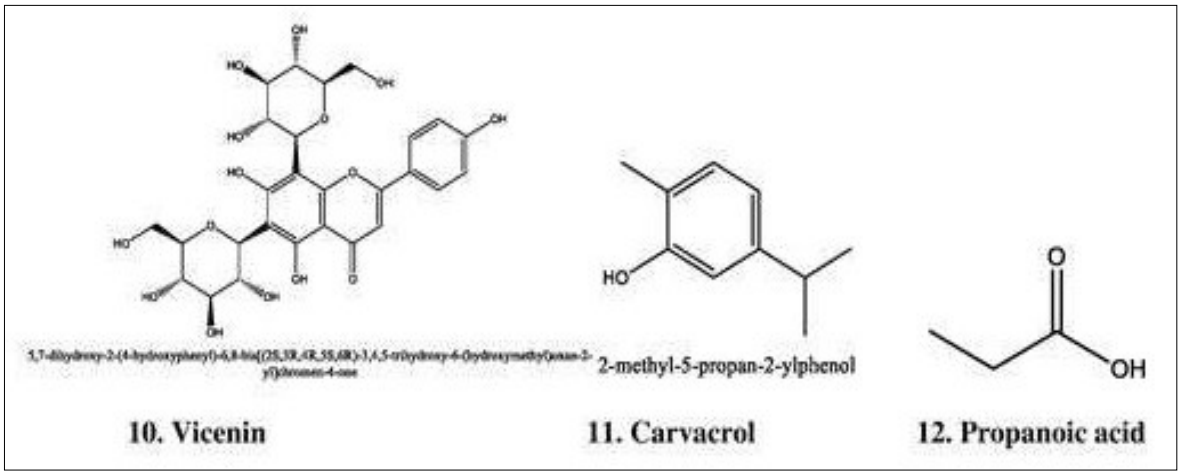
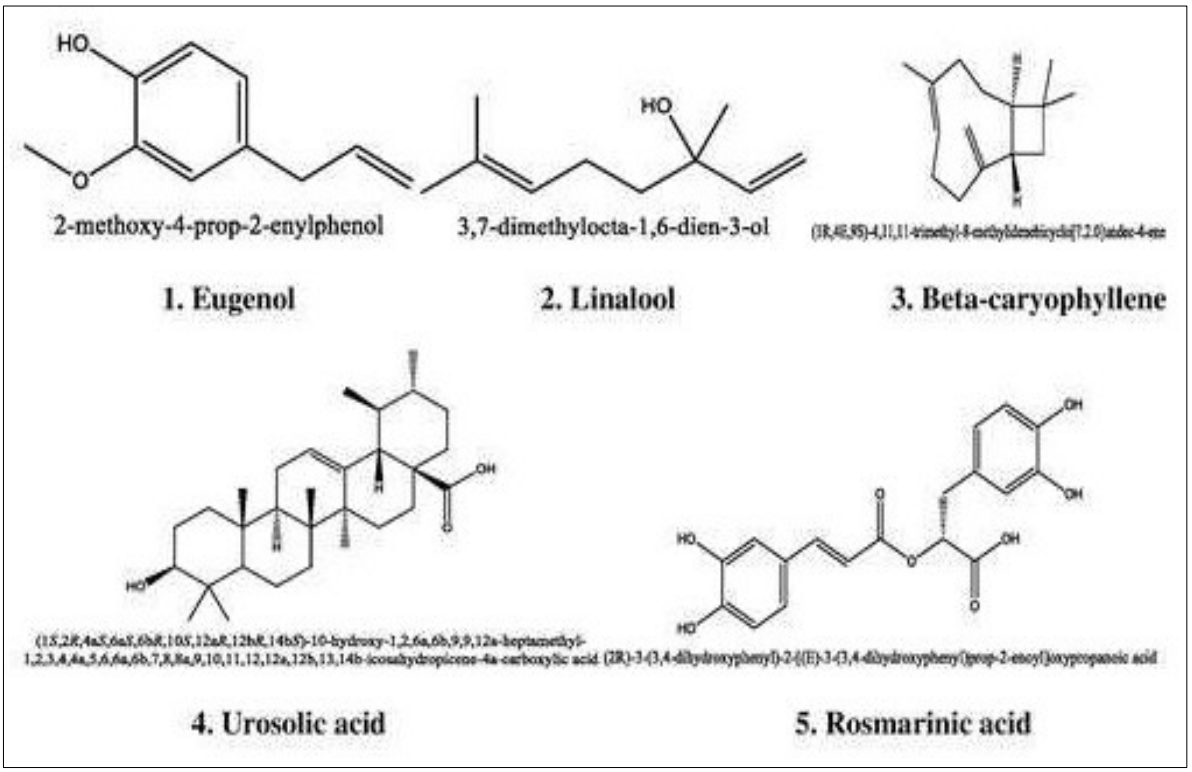
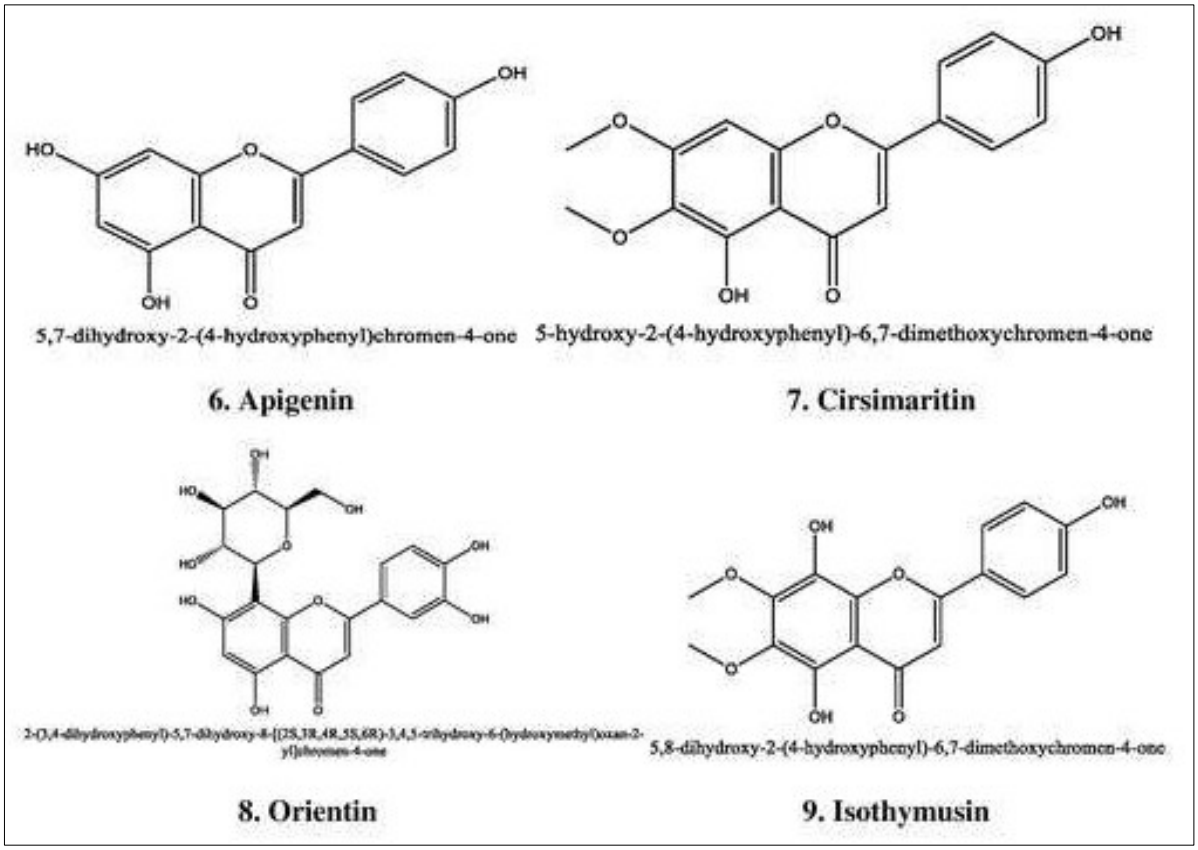


Figure 4: Structures and IUPAC Names of 1–9 Bio Molecules of Ocimum Sanctum Plant



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5.0 Conclusions

The synergistic AYUSH-based formulation prepared from Ocimum sanctum and Solanum xanthocarpum demonstrated potent immunomodulatory, antioxidant, and anti-inflammatory properties with excellent safety profile. The formulation effectively regulated cytokine responses and enhanced immune activity without causing toxicity. These findings support the potential application of SABF as a safe herbal therapeutic for management of paraTB infection and related inflammatory diseases.

The present investigation highlights the immunotherapeutic potential of SABF against paraTB infection. Enhanced splenocyte proliferation and cytokine regulation indicate activation of cell-mediated immunity essential for controlling intracellular pathogens such as MAP.

Ursolic acid and solasodine are known to modulate inflammatory mediators and improve macrophage activation. The observed decrease in TNF-α may help reduce chronic intestinal inflammation associated with MAP infection. Antioxidant activity further contributes to protection against oxidative stress induced during chronic infection. The absence of toxicity supports the traditional medicinal use of these herbs. Herbal formulations often provide multitarget therapeutic action with reduced side effects compared to synthetic drugs.

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