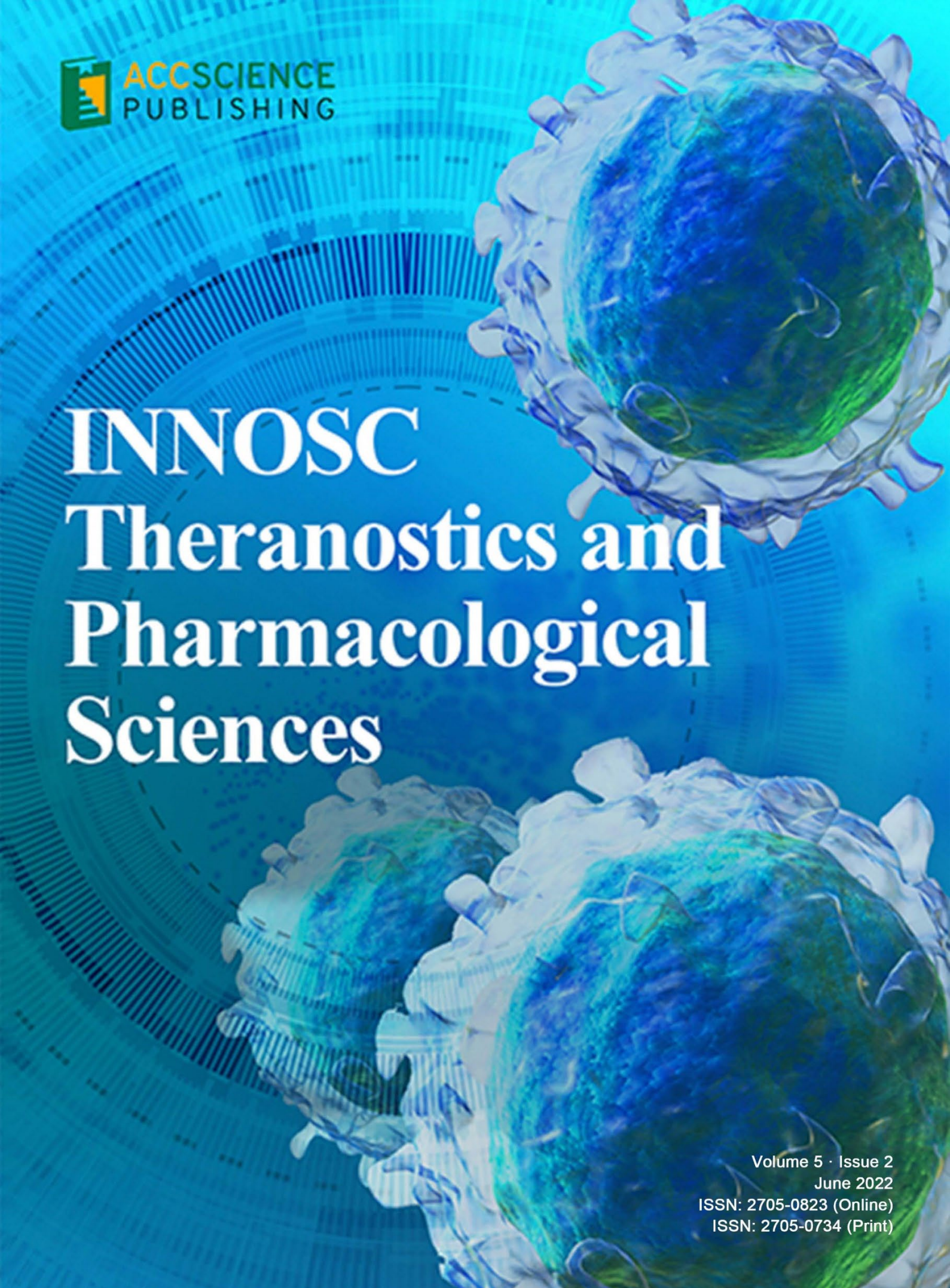

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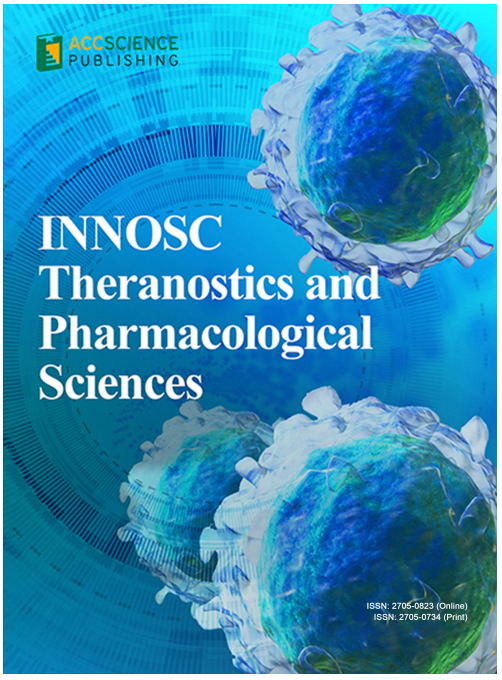

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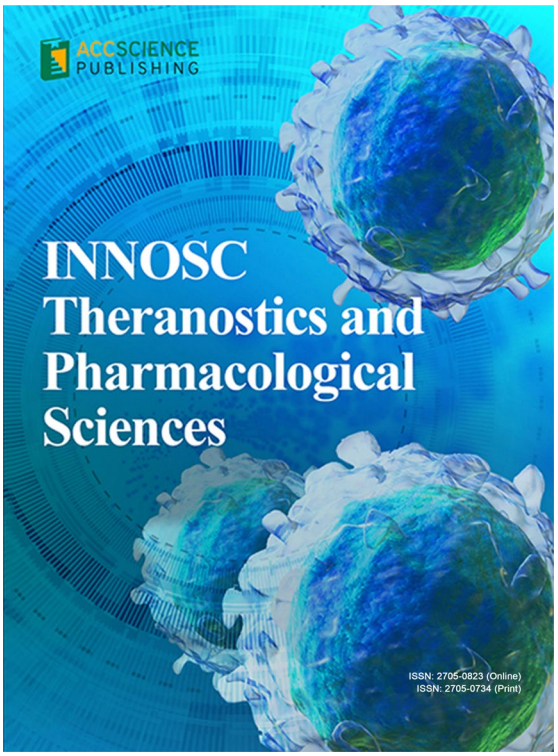

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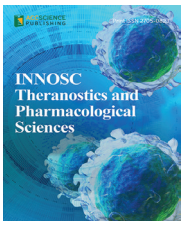

REVIEW ARTICLE

Agmatine as a Novel Treatment Option for Neuropathies: Experimental Evidences

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Abstract:

N-methyl-D-aspartate receptor (NMDAR) antagonist, a subclass of glutamate receptors or nitric oxide synthase (NOS) inhibitors, prevents neuronal plasticity. However, neural plasticity plays a major role in the pain caused by inflammation and neuropathy, providing clinical opportunities for the use of NOS inhibitors and NMDAR antagonists in the treatment of chronic pain. The neuromodulator agmatine has both NOS inhibitory and NMDAR antagonistic activity, and it controls a range of neurotransmitters and signaling pathways in the brain and spinal cord. The effects of agmatine on pain modulation are described and explored in this article, along with a potential mechanism of action for these effects. We specifically offer evidence to support further clinical and pre-clinical trials looking into agmatine as a novel therapeutic agent for neuropathic pain.

Keywords: Agmatine, Neuropathic pain, Nitric Oxide, N-methyl-D-aspartate

1. Introduction

Allodynia and hyperalgesia are a part of neuropathic pain (NP), a type of chronic pain brought on by a lesion or illness that affects the somatosensory system in the peripheral or central nervous system (CNS) [1]. Damage to the peripheral nervous system (PNS) caused by infections, some medicines, metabolic illnesses, and mechanical trauma are the main causes of NP. Numerous mechanisms, including alterations in gene expression and adjustments to ion channels that result in ectopic activity in the PNS, are thought to contribute to NP. In the CNS, numerous gene controls may also be changed. There is an alteration in afferent inputs of both nociceptive and non-nociceptive pathways due to neuronal death and excessive synaptic activity [2]. Research on animal models of peripheral nerve injury suggests that N-methyl-D-aspartate (NMDA) receptors (NMDARs) have a function in NP [3]. According to some studies, treatment of NMDAR antagonists eliminates the impulsive pain and the thermal and mechanical hyperalgesia induced in rats with loose sciatic nerve ligation. The study also implies that inhibiting NMDAR delays pain-related behavior after nerve injury [4]. It has been demonstrated that activation of NMDAR in different parts of the CNS causes nitric oxide generation, which, in turn, causes a Ca^{2+}-dependent rise in cyclic guanosine monophosphate (cGMP). The NMDAR is activated, which increases intracellular Ca^{2+} and

triggers nitric oxide synthase (NOS) to generate nitric oxide from free L-arginine. In this process, nitrate and nitrite are stable metabolites of nitric oxide, and monitoring their concentrations is an excellent way to assess the homeostasis of nitric oxide [5]. According to immunohistochemistry research, NOS-positive cells have been discovered in several regions of the animal and human brains, including the cerebellum and brainstem [6]. Studies suggest that sympathetic blockade caused by surgical and chemical agents reversibly reduces NP symptoms and that sympathetic efferent activity plays a significant permissive role in the emergence of hyperalgesia and allodynia in humans and animals suffering from NP [7].

Agmatine is an endogenous neurotransmitter that is produced in the mammalian brain and other tissues. It is the decarboxylated form of L-arginine and an NMDAR antagonist. It is an inhibitor of NOS, and it binds to α2-adrenoceptors, imidazoline (I1/I2) receptors, and serotonin (5-hydroxytryptamine or 5-HT) receptors with lesser affinity than NMDAR [8-10] (Figure 1). Studies have reported that agmatine is effective at reducing hyperalgesia and/or allodynia in a variety of chronic NP models when administered spinally and systemically. Agmatine also significantly reduces inflammation-related pain in animal models [11-13]. Agmatine has been shown to improve opioid analgesia and lessen diabetic neuropathy in rats, despite the fact that it does not appear to be an effective analgesic for acute phasic pain. According to a recent clinical investigation, agmatine is effective and safe for relieving pain and enhancing the quality of life in individuals with lumbar disk-associated radiculopathy. Pyramidal cells in the rat hippocampus and other parts of the brain contain agmatine [14]. Numerous physiological, pharmaceutical, and behavioral research suggests that hippocampus formation is crucial for the effective and motivating aspects of pain perception. The role of agmatine in the primary sense of pain has been reported [15,16]. After a peripheral nerve lesion, the proinflammatory cytokine tumor necrosis factor-alpha (TNFα) was markedly elevated in the hippocampus, and its administration into the hippocampus led to NP-like symptoms [17,18]. In this review, the studies indicating how agmatine affects NP are examined along with the underlying mechanisms of action. We also provide data to support clinical trials that would demonstrate agmatine's effectiveness as an NP adjuvant or monotherapy.

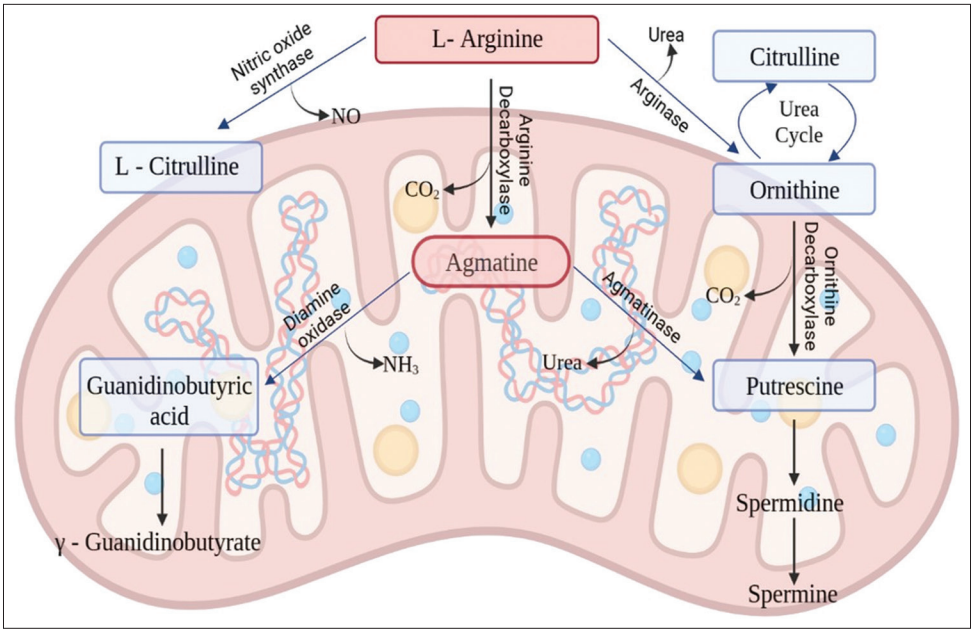

Figure 1. Schematic representation of biosynthesis and metabolism of agmatine inside the mitochondria of astrocytes. L-arginine acts as precursor molecule, which on decarboxylation by the enzyme arginine decarboxylase, coverts to agmatine. Agmatine is then metabolized to guanidinobutyric acid by the enzyme diamine oxidase.

2. Etiology

NP is associated with various medical conditions, the causes of which vary considerably. Metabolic (hypothyroidism), compressive (carpal tunnel syndrome), infectious (huma immunodeficiency virus, HIV), toxic (alcohol), traumatic (spinal cord injury), and ischemic (diabetes) processes are all causal factors of NP [19]. Multiple NP mechanisms may be linked to a single medical issue. In postherpetic neuralgia, viral, inflammatory, and ischemic processes have been discovered. Understanding the fundamental causes of NP may lead to advancements in classification, diagnosis, and treatment [20].

3. Classification of NP

Pain can be classified into neuropathic (pain caused by damage to the nervous system) and nociceptive (pain caused by stimulation of peripheral nociceptors in the skin, joints, and muscles). The primary distinction between NP and nociceptive is that NP is characterized by a lack of constant nociceptive input. Peripheral and central NP is the two categories into which NP is categorized [21]. A lesion or dysfunction causes peripheral NP in the PNS, such as postherpetic neuralgia, diabetic neuropathy, or causalgia. Thalamic pain, post-stroke pain, and post-spinal cord injury pain are examples of central NP that are linked to CNS damage or dysfunction. In addition, NP can either be induced by or unaltered by stimuli. Hyperalgesia and allodynia brought on by mechanical, thermal, or chemical stimulation are what set stimulus-evoked pain apart. Chronic or episodic, stimulus-independent (spontaneous) pain is usually described as shooting, burning, or stabbing [22].

4. Pathophysiology

There is currently no conclusive pathophysiology for NP. It is impossible to predict the underlying mechanisms based on a patient's etiology of the neuropathy, pain distribution, or symptoms. There are several hypothesized mechanisms of NP. NP can develop when there is injury or pathological damage to the PNS or CNS [23]. To summarize **(Figure 2)**, the understanding of NP, increased peripheral nerve firing, decreased CNS inhibition, and altered CNS processes result in pain amplification and propagation.

5. Pharmacological treatment for the management of NP

The management of NP is quite challenging. Despite taking the recommended medications for their diseases, patients with NP will still generally suffer from moderate to severe pain [23,24]. Therefore, recommendations for the pharmaceutical therapy of NP must be supported by data. Numerous drugs have consistently demonstrated efficacy in meta-analyses and randomized controlled clinical studies, including antidepressants with serotonin reuptake and norepinephrine inhibitors, Ca^{2+} channel α2-δ2-ligands, opioid analgesics, and topical analgesics such as lidocaine [25]. The mode of action, information on dosage, and side effects for different medication classes are summarized in **Table 1**.

6. Preclinical animal models of NP

The high degree of variability in the etiology of NP, which can be caused by conditions like diabetes mellitus or anti-cancer chemotherapy, as well as brain lesions, demyelinating diseases of the spinal cord, and peripheral neuropathy, is a major concern for research into and treatment of this condition [32]. Although the case histories of most NP patients do not resemble those of preclinical studies, they do allow for the investigation of disease processes, and many clinically used treatments can reverse the pain behaviors seen in these models [33]. The majority of preclinical studies are carried out on rodents and involve direct nerve injury to the sciatic nerve. Animal models that mimic the etiology of human diseases, such as diabetic neuropathy and some chemotherapy medications, are available in addition to surgical models [34,35]. However, due to inefficiency or adverse effects, it has long been difficult to effectively translate painkillers from animal models to clinical studies [36,37]. Due to the highly variable etiology of NP in humans compared to the relatively constrained animal models, the inbred (and thus genetically homogeneous) animal subjects in comparison to the more genetically diverse human population, and the difference in how pain is assessed (i.e., most animal studies measure

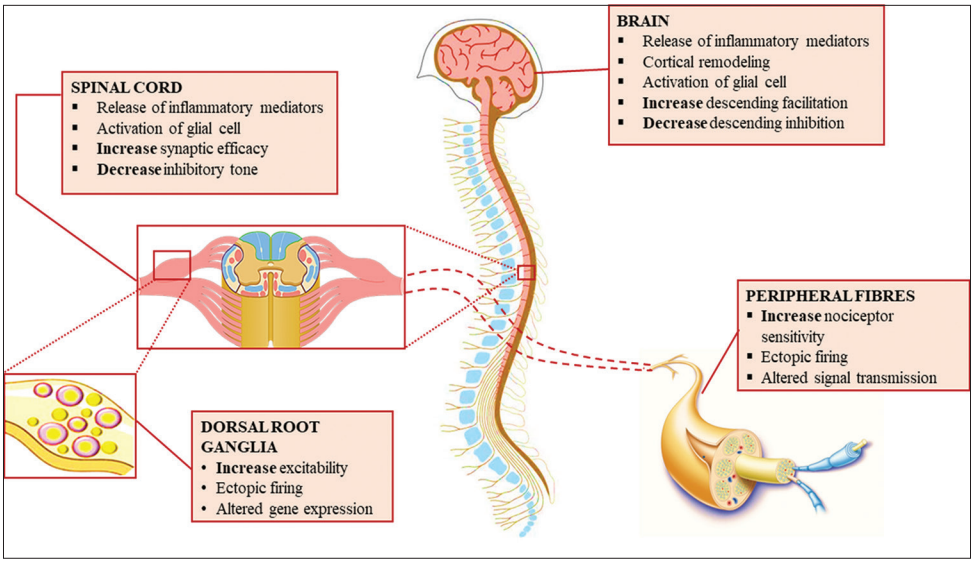

Figure 2. Diagrammatic representation of neuropathic pain pathophysiology in the brain and spinal cord.

stimulus-evoked withdrawal behaviors, whereas NP patients frequently describe spontaneous pain), among other differences. Animal models of NP have their limitations, and translation is far from perfect at this time, but these models have shown that NP involves changes at all levels of the pain pathway, from variations in the expression of the sensory neuron protein to changes in spinal cord synaptic function and the brain's descending control of pain [25,37]. Some of the studied animal models of NP are described in **Table 2**.

7. Agmatine as a potential treatment for NP

Dysesthesia or pain that results from stimuli that are generally non-painful (allodynia) are two characteristics of NP, which is caused by injury to or disease of the somatosensory nervous system [53,54]. Agmatine has been demonstrated to lessen pain-related behaviors in rodent models of neuropathic sensory hypersensitivity [55,56]. In contrast to morphine-based analgesics, this action is mediated by NMDAR regulation and nitric oxide signaling. Concerning the latter, it has been demonstrated that agmatine increases the analgesic efficacy of morphine through the α2-adrenoceptor-mediated mechanism [8,57,58]. It has been demonstrated that agmatine inhibits the production of advanced glycation end-products and enhances nerve regeneration in diabetic neuropathy [59]. A placebo-controlled clinical experiment showed that agmatine treatment might be efficient in humans as well. Agmatine produced anti-allodynic and anti-hyperalgesic benefits in animal models of chronic neuropathic and inflammatory pain [14]. Acute delta opioid receptor-mediated analgesia was potentiated and enhanced in response to intrathecal agmatine administration. Chronic pain is a typical side effect of traumatic nerve injury, which can also activate NOS, NMDA, and other common pathogenic cascades. NOS produces nitric oxide from L-arginine when NMDAR activation results in an increase in intracellular Ca^{2+}. It is generally known that NMDAR is crucial in the processing of chronic pain brought on by injury to peripheral nerves. A decrease in nitric oxide levels and noradrenergic activity in the brain may have contributed to agmatine's attenuation of the sciatic nerve ligation-induced NP model. These beneficial effects of agmatine may be attributed to the engagement of noradrenergic neurons in the locus coeruleus, which are implicated in the development and/or maintenance of allodynia and hyperalgesia in the context of peripheral nerve injury. Agmatine has the ability to bind to both the α2-adrenergic and imidazoline receptors [60]. One hypothesis was that an imbalance of supraspinal facilitation and inhibition constituted the root of neuropathic hypersensitivity. The locus coeruleus may be involved in the bidirectional control of pain [31]. Lesions in the noradrenergic locus coeruleus were shown to suppress the onset of allodynia and hyperalgesia, while noradrenergic reuptake inhibitors were found to reduce NP. Even though it

Table 1. First-line treatment for patients with neuropathic pain

Mechanism of action		Dose (starting/ maximum)	Side effects
Selective serotonin-norepinephrine reuptake inhibitors (SSNRIs)			
Venlafaxine [26]	Inhibits both norepinephrine and 5-HT reuptake	37.5 mg (once or twice daily)/225 mg (daily)	Nausea
Duloxetine [27]	Inhibits both norepinephrine and 5-HT reuptake	30 mg (once daily)/60 mg (twice daily)	Nausea
Tricyclic antidepressants (TCAs) [28]			
Desipramine Nortriptyline	Inhibits the reuptake of norepinephrine and/or 5-HT, blocks Na^+channels, anticholinergics	25 mg (bedtime)/150 mg (daily)	Anticholinergic effects (dryness of mouth, weight gain, urinary retention, sedation)
Topical lidocaine [29]			
5% lidocaine patch	Blockade of Na^+channels	1–3 patches/3 patches	Rash, local erythema
Calcium channel α2-δ ligands [30]			
Pregabalin	Decreases release of norepinephrine, glutamate, and substance P, with ligands on the α2-δ subunit of voltage-gated calcium channel	50 mg (thrice daily or 75 mg twice daily)/200 mg (thrice daily) or 300 mg (twice daily)	Sedation, peripheral edema, dizziness
Gabapentin	Decreases release of norepinephrine, glutamate and substance P, with ligands on the α2-δ subunit of voltage-gated calcium channel	100–300 mg (once to thrice daily)/1200 mg (thrice daily)	Sedation, peripheral edema, dizziness
Opioid agonists [31]			
Tramadol	Agonist of μ-receptor inhibits the reuptake of norepinephrine and serotonin	50 mg (once or twice daily)/400 mg (daily as a long-acting drug)	Vomiting/nausea, constipation, dizziness
Morphine, oxycodone, methadone, levorphanol	μ-receptor agonist (oxycodone also causes κ-receptor antagonism)	10–15 mg morphine every 4 h or as needed (equianalgesic doses should be used for other opioids)/no maximum doses	Vomiting/nausea, constipation, dizziness

5-HT: 5-hydroxytryptamine (serotonin)

seems like the locus coeruleus which is a structure that prevents pain, some studies indicate that it may actually have a function in NP facilitation. There has been speculation that the coeruleospinal noradrenergic fibers are involved in the descending inhibition of spinal pain transmission.

Agmatine has been demonstrated to lower 3-methoxy-4-hydroxyphenyl ethylene glycol (MHPG) and norepinephrine levels in the brainstem while increasing the norepinephrine (NP) pain threshold. It was suggested that the reduction of NP was due to agmatine's activation of presynaptic α2-adrenoceptors, which reduced central noradrenergic activity. The oxidative and nitrosative stress elicited by persistent hyperglycemia in diabetes mellitus is regarded as one of the key factors in the disease's related brain dysfunction. Increased oxidative stress promotes vascular dysfunction and endoneurial hypoxia, which impairs the function of the motor and sensory nerves. In addition, rats with diabetes caused by streptozotocin were also found to have an L-arginine shortage. Nitric oxide, agmatine [61], and glutamate all have similar effects on the CNS through

Table 2. Animal models of neuropathic pain

No.	Name of animal model	Principle	Species
1.	Axotomy (complete sciatic nerve transection) [33,38]	Whole transection of sciatic nerve	Rats
2.	Acrylamide-induced injury [39]	Prolonged administration of acrylamide	Rats
3.	Chronic constriction injury [33,40]	Four loose ligatures around the sciatic nerve	Rats, mice
4.	Partial sciatic nerve ligation (Seltzer Model) [41,42]	Tight ligation of one-third to half of the sciatic nerve	Rats, mice
5.	Orofacial pain [43]	Injection of formalin, carrageenan into temporomandibular joints and maxilla	Rats, mice
6.	Spinal nerve ligation [44,45]	Tight ligation of L5 and L6 spinal nerves	Rats
7.	Spared nerve injury [46,47]	Axotomy of tibial and common peroneal nerves	Rats, mice
8.	Trigeminal neuralgia [48,49]	Compression of trigeminal ganglion; chronic constriction injury to the infra-orbital nerve	Rats Rats
9.	Diabetes-induced neuropathy (streptozotocin-induced andgenetic models) [50,51]	Persistent hyperglycemia-induced changes in the nerves	Rats, mice
10.	Weight drop or contusive spinal cord injury [52]	Dropping a weight over the exposed spinal cord	Rats, mice

NMDAR [61]. Agmatine likely works on spinal imidazoline receptors to reduce pain [14]. Agmatine was reported to have lessened the antiallodynic and anti-hyperalgesic effects in diabetic NP when combined with an imidazoline receptor antagonist. In spinal nerve ligation animal modelswith diabetic NP, agmatine also exerts an antiallodynic effect. When all underlying causes of NP are taken into account, agmatine can substantially treat numerous neuropathies owing to its NMDAR antagonist, NOS inhibitory, and anti-inflammatory effects. One of the pathogenic pathways that can be triggered by neuronal injury and chronic pain is the activation of NMDAR and NOS [62]. All isoforms of NOS, especially the most powerful types, as well as NMDAR and NMDA-mediated Ca^{2+} currents, have all been reported to be blocked by agmatine **(Figure 3).**

It has recently been reported that cisplatin-induced mechanical allodynia, sciatic nerve degeneration [63,64], and dorsal root ganglia cell senescence may be prevented by agmatine. Studies indicate that agmatine's neuroprotective and antiallodynic effects were not significantly increased by L-NAME. Another study demonstrated that NOS inhibitors and NMDAR antagonists [65] can activate tryptophan hydroxylase to enhance the release of 5-HT. Furthermore, it was suggested that the production of neurotrophic chemicals by macrophages, activated microglia, and infiltrating monocytes contribute significantly to neuroinflammation. These compounds have both pro-inflammatory and neuroprotective effects. It was suggested that agmatine could enhance the anti-inflammatory M2 macrophage properties without boosting cell numbers. Its anti-neuropathy effectiveness may possibly be due to the proinflammatory M1 and anti-inflammatory M2 macrophages' induction of the activation of axonal regeneration after neuronal damage. The hippocampus is one of the many regions of the brain where agmatine is widely distributed and colocalizes with sigma receptors. Sigma receptors were present in sciatic nerves [66,67] as well, and Sigma-1 receptors, in particular, were crucial for controlling NP. Furthermore, some data suggest that NP may have higher-than-normal hippocampal TNFα levels [68]. Sigma-1 and Sigma-2 receptor [63] agonists were shown to boost TNFα production, whereas agmatine lowered TNFα levels, indicating that inhibiting these receptors in NP-induced rats may be beneficial. As a result, the antinociception induced by agmatine may include the serotonergic,

opioidergic [69-72], imidazoline [11,73,74], α2-adrenergic [75,76], and opioidergic sigma receptors, which have been recently shown to have a substantial role in the antinociceptive effect of agmatine on NP. Hence, additional research is required on these predictions **(Table 3).**

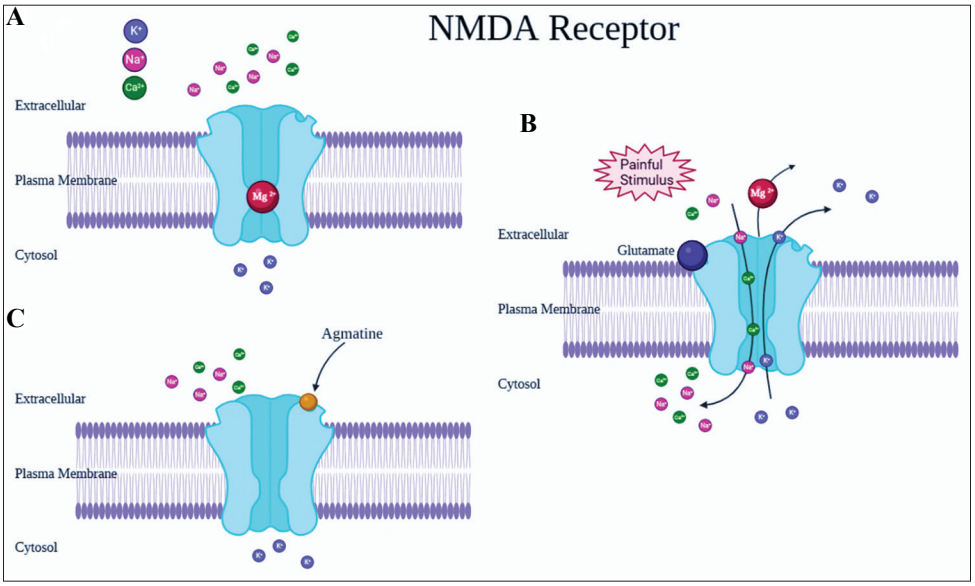

Figure 3. Effect of agmatine on N-methyl-D-aspartate (NMDA) receptor. (A) At the resting stage, NMDA receptor is blocked by magnesium ion. (B) During painful stimulus, there is release of an excessive amount of glutamate ions, which over-activate the receptor and transport the ions in and out, leading to Ca^{2+} excitotoxicity. (C) Agmatine, an NMDA receptor antagonist, binds to the receptor and attenuates the Ca^{2+} ions excitotoxicity.

Table 3. Experimental evidence for the analgesic effect of agmatine on different animal models of neuropathic pain

Behavioral test	Neuropathic pain induction	Route of administration	Dose	Effect
Von Frey test [77]	Spinal nerve ligation	i.t.	0.3 nmol	+
Von Frey test [77]	i.t. Dynorphin	i.t.	0.3 nmol	+
Cold hyperalgesia [78]	Sciatic nerve ligation	i.p.	10 – 100 mg/kg	+
Thermal hyperalgesia [7]	Sciatic nerve ligation	i.p.	30 – 400 mg/kg	+
Von Frey test [79]	Spinal nerve ligation	i.p.	10 – 100 mg/kg	+
Von Frey Test [79]	Diabetic neuropathy	i.p.	10 – 100 mg/kg	+
Plantar test [10]	Diabetic neuropathy	i.t.	4.4 – 438 nmol	+
Von Frey test [10]	Diabetic neuropathy	i.t.	4.4 – 438 nmol	+
Paw pressure test [10]	Diabetic neuropathy	i.t.	4.4 – 438 nmol	+
Cold hypernociception [80]	Partial sciatic nerve ligation	i.p.	30 mg/kg	+
Thermal hypernociception [54]	Partial sciatic nerve ligation	i.p.	30 mg/kg	+
Cold allodynia Thermal allodynia Mechanical allodynia [12]	Partial sciatic nerve ligation	i.h.	2.5 – 10 µg/side/rat	+
Cold allodynia Thermal allodynia Mechanical allodynia [12]	Partial sciatic nerve ligation	i.p.	40 – 120 mg/kg	+

i.h: Intrahippocampal; i.p: Intraperitoneal; i.t.: Intrathecal; +: Analgesic effect.

8. Conclusions

Multiple, intricate mechanisms lead to the emergence of NP. Animal models were used to better define the therapy objectives and to comprehend the pathophysiological mechanisms. Given that agmatine has numerous targets that are likewise implicated in NP and open up novel therapeutic possibilities, there have been numerous scientific studies on the impact of agmatine on NP. Agmatine has a high safety profile and has not been demonstrated to negatively affect behavior, locomotion, or cardiovascular systems in naive animals. According to experimental investigations, agmatine has shown considerable potential for the development of effective treatment for this illness.

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Conflict of interest

The authors declare no conflicts of interest.

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Ethics approval and consent to participate

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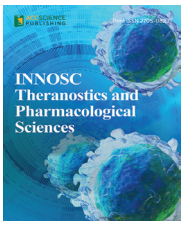

CASE REPORT

Non-Hospital-Acquired *Pseudomonas aeruginosa* Keratitis in a 7-Month-Old Infant

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Abstract:

Pseudomonas aeruginosa is one of the important pathogens causing bacterial keratitis with ulceration. In this study, we evaluated a case of non-hospital-acquired *P. aeruginosa* keratitis in a 7-month-old infant and its treatment process. The patient who had no known chronic disease was brought in with complaints of watering and redness in the left eye. The complaint of the eye problems started 1 month ago after the patient swam in a farm pool. Keratitis was diagnosed according to an ophthalmologic examination in our clinic. Left corneal scraping cultures were taken for the identification of the pathogen. Moxifloxacin eye drops and ganciclovir gel were prescribed for 10 days. The corneal haze disappeared after the treatment. Reasons for delay in diagnosis and treatment were evaluated in our case. We also compared the keratitis between adults and infants. *P. aeruginosa* keratitis should be considered in the differential diagnosis, taking into account the presence of resistance to treatment, visual disturbances, lens usage, nasolacrimal duct obstruction, trauma, and bad hygiene. Congenital nasolacrimal duct obstruction is an important risk factor for infants. Vision loss, which may happen in adults, cannot be clearly communicated by infants due to their limited communication abilities. *P. aeruginosa* should be considered the pathogen causing keratitis in infant patients, particularly when the diagnosed characteristics align with our case.

Keywords: Keratitis, *Pseudomonas aeruginosa*, Ulceration, Infant, Infection

1. Background

Infectious keratitis is a major cause of visual impairment and blindness in adults [1]. Infections are still predominant and are found in 80% of patients with ulceration [2]. One of the important pathogens of bacterial keratitis with ulceration is *Pseudomonas aeruginosa* which is often associated with contaminated contact lenses, eye trauma, and hospitalization history [3]. With various metabolic pathways and a vast repertoire of pathogenic mechanisms, this Gram-negative bacillus can survive a broad range of environmental conditions [4]. Treatment of *P. aeruginosa* keratitis may be difficult because this bacterium can resist antibiotics through intrinsic and acquired mechanisms such as the transfer of resistance through interchangeable genetic elements. A large variety of virulence factors contribute to its importance in burn wounds, lung infections, and eye infections, including pili, flagella, lipopolysaccharide, proteases, quorum sensing, exotoxin A, and exoenzymes secreted by the type III secretion system [5].

Rapid diagnosis and treatment of bacterial keratitis are essential to limit stromal scarring and minimize potential vision loss. Thus, treatment should be started empirically early [6]. This pathogen is mostly seen in adults due to contact lens usage [7]. However, the most important risk is hospitalization for infants. In the literature, *P. aeruginosa* keratitis in infants is frequently hospital-acquired. Cases of non-hospital-acquired *P. aeruginosa* keratitis in infants are especially rare.

2. Case presentation

A 7-month-old female infant who had no known chronic disease was brought in with the complaint of watering and redness on the left eye. One month prior, the patient swam in a pool on a farm, and then the complaints started. At that time, nasolacrimal duct obstruction was considered by a physician at the ophthalmologic examination, and the physician prescribed some eye drops and suggested waiting 1 year. The patient has used netilmicin and dexamethasone drops during this time. When the patient came to our clinic again, her complaints did not decrease. According to ophthalmologic examination results, the patient was diagnosed with keratitis. Macroscopically, the right eye appeared normal, and the anterior and posterior chambers of the right eye were also normal. The left eye conjunctiva was less red than in a keratitis case (**Figure 1)**.

The lesion in the cornea was not wide. Watering was observed in the left eye. Keratitis focuses were observed to prevent retinoscopy reflections in the cornea, especially on the central cornea and the lower half of the cornea. The anterior chamber, pupil, and lens looked normal. The fundus was not enlightened.

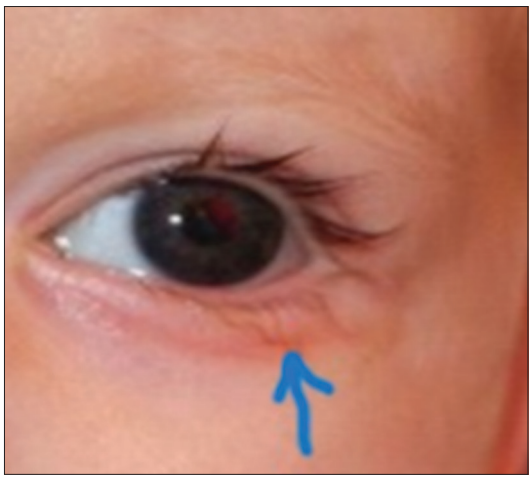

Figure 1. Pre-treatment corneal appearance.

Left corneal scraping material and cultures were taken. Gram stains of the samples from the conjunctiva and cornea were performed. Abundant leukocytes and Gram-negative bacillus were seen at the microscopic examination on Gram stains.

All samples were inoculated with 5% sheep blood, eosin methylene blue (EMB) agar, and chocolate agar containing Poly ViteX for aerobic bacterial cultures and were incubated at 37°C for 24–48 h. Samples of EMB and 5% sheep blood agar cultures from the left conjunctiva and left cornea produced typical *P. aeruginosa* (**Figure 2**). This pathogen was found to be susceptible to amikacin, ceftazidime, levofloxacin, ciprofloxacin, piperacillin, and tazobactam. Gentamicin eye drop was added to the treatment on the 4th day of the treatment.

The produced *P. aeruginosa* was detected using API 10 S Gram-negative identification kit (Biomerieux, ABD). Antibiotic susceptibility tests were performed with disc diffusion (**Figure 3**). Clinical and Laboratory Standards Institute criteria

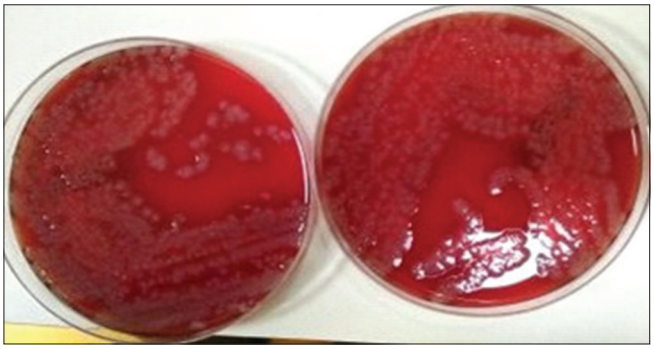

Figure 2. Bacterial culture on 5% sheep blood agar from the left corneal scraping.

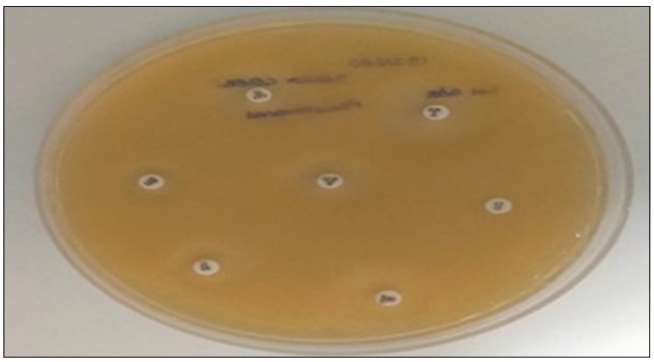

Figure 3. Muller–Hinton agar plate showed antimicrobial susceptibility profile of the pathogen.

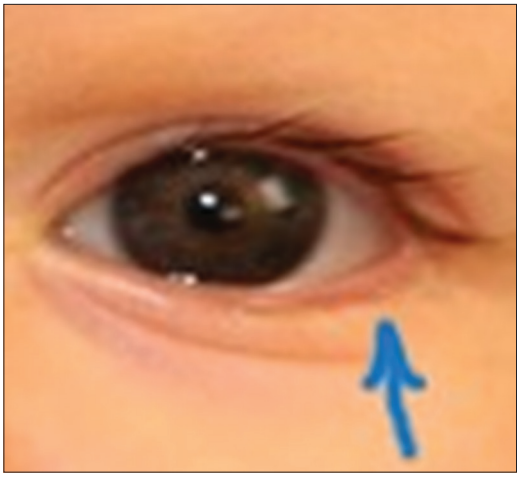

Figure 4. Corneal appearance after treatment.

were used in the evaluation of antimicrobial resistance patterns.

As for the treatment regime, moxifloxacin eye drops and ganciclovir gel were prescribed. The corneal haze disappeared on the tenth and final day of the treatment (**Figure 4**).

Congenital nasolacrimal duct obstruction may have been a predisposing factor for the patient. Early probing was suggested to the patient.

3. Discussion

In the presented case, the isolated microorganism is associated with pathogenic keratitis, which plays a crucial role in keratitis. Environmental factors such as poor hygiene and trauma are important risk factors for keratitis. In the literature, keratitis cases caused by *P. aeruginosa* are usually associated with contact lens usage. This pathogen is rarely reported in infants without hospitalization history. As infants can not describe their complaints, diagnosis in infants can be delayed. Antibiotic resistance is a significant factor contributing to treatment delays. If treatment is delayed, this pathogen can invade the cornea, anterior chamber, and aqueous humor.

Congenital nasolacrimal duct obstruction is one of the risk factors for infants [8]. Data published by Li *et al*. shows that human tear fluid can protect against *P. aeruginosa*, the major opportunistic pathogen, independently of its bacteriostatic activity [9]. In our case, the patient had nasolacrimal duct obstruction. Furthermore, the patient had a story of swimming in a pool at a farm. We considered that the pathogen infected the patient with a nasolacrimal duct obstruction risk factor through the contaminated pool. The fact that the pathogen is susceptible to so many antibiotics also supports our opinion that it is environmentally acquired.

The intact cornea is normally resistant to invasion by *P. aeruginosa* [10]. Probably, nasolacrimal duct obstruction facilitated corneal trauma. Furthermore, the patient had a story of using corticosteroid drops. All of these reasons caused the patient to be susceptible to polymicrobial keratitis. As in the case of Hue *et al*., if the pathogen invaded the anterior chamber, systemic anti-biotherapy was needed [11]. However, local treatment was enough because there was no sign of invasion in our case.

4. Conclusion

P. aeruginosa keratitis is a disease that should be treated seriously, as it causes visual impairment if diagnosis is delayed. *P. aeruginosa* keratitis should be considered in the differential diagnosis, particularly for infants, when there are risk factors such as poor hygiene, contact lens usage, treatment-resistance keratitis, visual disturbances, and nasolacrimal duct obstruction identified in the differential diagnosis.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Keramettin Yanik
Formal analysis: Hatice Buse Uras, Celal Yeter
Investigation: Keramettin Yanık
Supervision: Celal Yeter
Writing – original draft: Hatice Buse Uras
Writing – review & editing: Hatice Buse Uras

Ethics approval and consent to participate

Informed consent was obtained from the study subject's guardians for participating in the study.

Consent for publication

Informed consent was obtained from the study subject's guardians for publishing the data.

Availability of data

Data can be obtained from the corresponding author following formal request.

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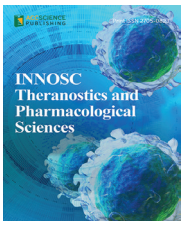

RESEARCH ARTICLE

Poor Adherence to Indications for Anti-neutrophil Cytoplasmic Antibody Testing in a South African Tertiary Hospital

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Abstract:

Appropriate use of laboratory investigations is increasingly important in resource-constrained environments. In this study, we reviewed the anti-neutrophil cytoplasmic antibody (ANCA) testing practices in a tertiary hospital in South Africa. A retrospective file review was conducted, encompassing all ANCA tests ordered over 12 months, including both inpatients and outpatients. Sociodemographic and clinical details were extracted from the patient records. All requests were assessed against the International Consensus Statement of 1999, which provides clinical guidelines for the indications for ANCA testing. Of the 945 ANCA tests requested, 790 patient records were reviewed, while 155 records were found to be missing, and 62 patients had multiple tests. Only 193 patients (24.4%) had indications for ANCA testing that met the guidelines. The most common tests done outside guideline indications were critical limb ischemia (9.6%), stroke (7.3%), uveitis (5.7%), renal impairment (4.9%), and interstitial lung disease (4.4%). Among the patients, ten (1.3%) were diagnosed with ANCA-associated vasculitis (AAV), of whom nine had renal-limited vasculitis. Twenty-six patients tested positive for ANCA without any evidence of AAV. Of these false positives, 10 (38.4%) were human immunodeficiency virus (HIV) positive, 3 (11.5%) had tuberculosis (TB), and 3 (11.5%) had other autoimmune diseases. The annual cost of ANCA tests amounted to ZAR274,046, with ZAR17,490 spent on duplicate testing and ZAR208,275 on non-indicated clinical conditions. The study revealed that ANCA testing was performed outside standard guidelines in three-quarters of requests, and duplicate testing was common, resulting in large cost implications. Chronic infections, such as HIV and TB, and autoimmune conditions accounted for half of the false-positive tests. The findings suggest that training of clinicians is likely to reduce unnecessary tests.

Keywords: Anti-neutrophil cytoplasmic antibody, ANCA-associated vasculitis, Gated testing

1. Introduction

Anti-neutrophil cytoplasmic antibodies (ANCAs) are important diagnostic tools for ANCA-associated vasculitis (AAV), a group of necrotizing small vessel vasculitis characterized by few or no immune deposits. Due to the high false-positive rate for indiscriminate ANCA testing, the International Consensus Statements of 1999 and 2017 provided clinical guidelines of indications for ANCA testing (**Table 1**) [1,2]. The aim of these guidelines is to increase the positive predictive value (PPV) of ANCA testing by limiting the test to patients with clinical features suggestive of AAV. It has been shown that ANCA tests have a PPV of 54% and a negative predictive value (NPV) of 99% in conventional clinical settings, but with the application of guidelines, PPV is increased to

Table 1. 1999 international consensus statement guidelines of indications for ANCA testing* [1]

i.	Glomerulonephritis, especially rapidly progressive GN
ii.	Pulmonary hemorrhage, especially pulmonary renal syndrome
iii.	Cutaneous vasculitis with systemic features
iv.	Multiple lung nodules
v.	Chronic destructive disease of the upper airways
vi.	Long-standing sinusitis or otitis
vii.	Subglottic tracheal stenosis
viii.	Mononeuritis multiplex or other peripheral neuropathies
ix.	Retro-orbital mass
x.	Scleritis

Note: *when there is no other obvious cause.

62% [3]. Encouragingly, studies show that AAV is seldom missed when testing is restricted to indications listed in the 1999 clinical guidelines [3].

AAV seems to be rare in African populations. Only a limited number of studies on ANCA antibodies or AAV in Africa have been reported, most likely due to a combination of a low index of suspicion of AAV and a lack of appropriate laboratory facilities [4]. Among a group of 60 West Africans, 6.7% of patients with chronic infections (tuberculosis [TB] and malaria) tested positive for anti-myeloperoxidase (MPO). In contrast, no ANCA antibodies were detected among healthy Africans [5].

The rising cost of laboratory investigations and equitable distribution of healthcare resources has become important issues worldwide, with both social and political implications. In South Africa, cost-effectiveness is a major criterion in developing clinical policies. We audited all ANCA tests ordered in a tertiary academic hospital to assess adherence to indications for testing, evaluate the diagnostic accuracy of the test, and review the positive ANCA test results, aiming to decrease hospital costs and avoid inappropriate specialist referrals. The Human Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town, approved the study (HREC reference number 443/2018).

2. Methods

A retrospective record review was performed on all ANCA tests ordered through the National Health Laboratory Services (NHLS) at a state-sector tertiary hospital over 12 months. Case records were reviewed, and clinical and demographic features (including self-reported ethnic group), the department requesting the test, and comorbidities were collated. The indication for each ANCA test was assessed against the International Consensus Statement of 1999.

The revised 2017 international consensus on ANCA testing recommends the use of high-quality immunoassays as the preferred first screening method for granulomatosis with polyangiitis (GPA) and microscopic polyangiitis [2], which remains unchanged in the 2020 consensus statement [6]. The recommendations were based on a multi-center European Vasculitis Study Group evaluation which demonstrated that these assays had a higher diagnostic performance in comparison to manual and automated indirect immunofluorescence [7].

While the consensus statement does not specify a particular type of immunoassay to be used, a recent review from the Netherlands has indicated that, for routine screening, diagnosis, and follow-up, quantitative assays measuring ANCA levels are associated with higher likelihood ratios and a more definitive diagnosis [8]. On the other hand, qualitative immunoassays can be used for confirmation and rapid testing [8], generally interpreted using a single cut-off value.

The NHLS utilized a fluorescence enzyme-linked immunosorbent assay sandwich immunoassay for ANCA testing. The specific method employed was the EliA IgG assay, performed on the Phadia 250 (Thermofisher). This *in vitro* qualitative measurement detects IgG antibodies directed against proteinase 3 (PR3) and MPO in human sera. Before analysis, the sample preparation includes allowing the serum to clot at room temperature, followed by centrifugation at 2200× g for 15 min. The serum is then drawn off, and aliquots are transferred into appropriately labeled tubes. The aliquot samples are immediately analyzed. The cutoff values (as per kit manufacturer) for anti-PR3 and anti-MPO are 1.9 U/ml and 3.4 U/ml, respectively.

Descriptive statistics and normally distributed variables are presented as means with standard deviations, and sensitivity, specificity, PPV, and NPV were calculated. Analysis was conducted using IBM SPSS Statistics V26.

3. Results

Of 945 ANCA tests performed, 790 clinical records were identified and reviewed, while 155 patient records were missing (**Figure 1**). Among the patients, 62 had undergone multiple tests, resulting in a total of 133 duplicate tests. Most tests were ordered for inpatients (63.0%). Overall, only 193 patients (24.4%) had indications that met the 1999 guidelines for ANCA testing. The remaining 597 (75.6%) did not meet the guidelines criteria.

The departments that requested the most ANCA tests were Medicine, Ophthalmology, Neurology, and Surgery. Notably, surgery had 98.9% of its tests ordered for non-guideline indications (**Table 2**). The most common clinical problems for which ANCA tests were ordered outside of guideline indications included critical limb ischemia (9.6%), stroke (7.3%), uveitis (5.7%), acute kidney injury (4.9%), and interstitial lung disease (4.4%). On the other hand, the most common guideline indications were glomerulonephritis (GN) (52.1%), peripheral neuropathy (19.0%), scleritis (10.5%), and cutaneous vasculitis (9.0%).

Ten patients (1.3%) were diagnosed with AAV over the 12 months, and these patients were predominantly female, of mixed racial ancestry, with a mean (SD) age of 54.5 (16.4) years (**Table 3**). One patient was diagnosed with ANCA-negative granulomatosis with GPA, and nine had renal-limited vasculitis (RLV) with pauci-immune necrotizing GN on renal biopsy and no extra-renal manifestations. Only five of the RLV patients were ANCA-positive (mostly MPO-positive).

Of all ANCA tests performed, 31 patients (3.9%) had tested positive for ANCA, of which 5 were true positives and 26 were false positives showing no evidence of AAV. Of these false positives, 11 (42.3%) were associated with chronic infections, such as human immunodeficiency virus (HIV), TB, and syphilis, while three (11.5%) had autoimmune diseases (**Table 4**). There was no explanation for the positive results in 13 patients (50%). In terms of drug-induced ANCA positivity, no commonly associated drugs, including anti-thyroid drugs, D penicillamine, sulfasalazine, allopurinol, cocaine, or TNF inhibitors, were implicated in the false-positive ANCA group. The sensitivity, specificity, PPV, and NPV for the total cohort were 50.0%, 96.7%, 16.1%, and 99.3%, respectively.

Of the 193 ANCA tests which met guideline indications for testing, 14 patients tested positive for ANCA, of which five were true positive, while the remaining nine were false positives, showing no evidence of AAV. When focusing only on patients who met the guideline indications for testing, the PPV improved, although it remained

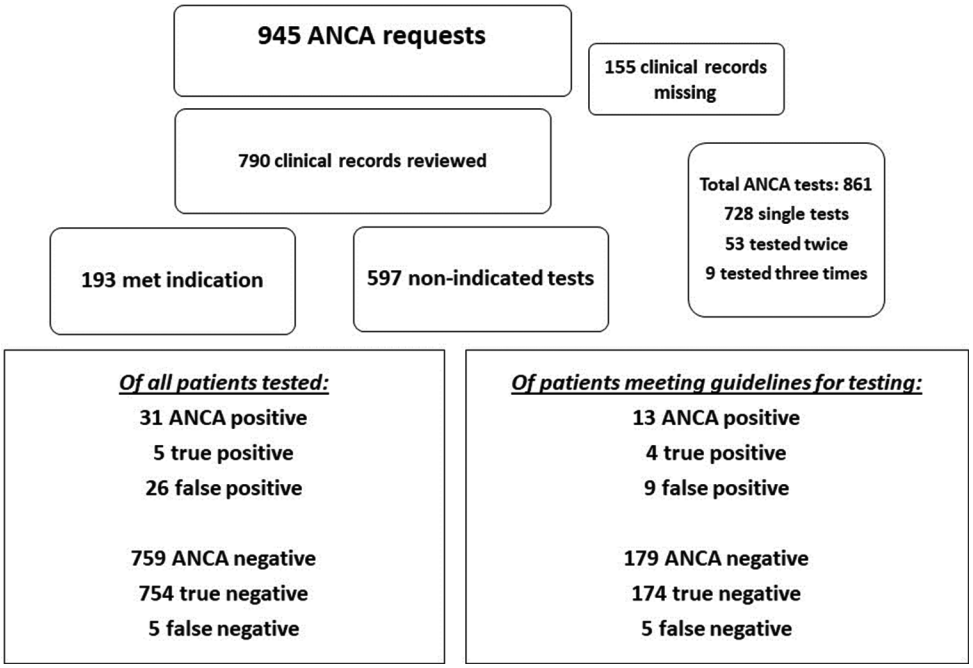

Figure 1. Flow diagram of anti-neutrophil cytoplasmic antibody tests performed over 12 months at a tertiary hospital in South Africa.

Table 2. ANCA testing according to indication guidelines per department

Department	Total requests *n*=790	1999 Guideline indication		Common non-guideline indications
		No *n*=598 *n* (%)	Yes *n*=192 *n* (%)	
Cardiology	4	4 (100)	0 (0)	Valvular heart disease
Critical care	22	15 (68.2)	7 (31.8)	AKI, seizures, encephalopathy
Dermatology	10	6 (60.0)	4 (40.0)	Non-specific rash
Otolaryngology	31	27 (87.1)	4 (12.9)	Sensory-neural hearing loss, vocal cord paralysis
Gastroenterology	3	3 (100)	0 (0)	IBD
General Medicine	272	173 (63.6)	99 (36.4)	AKI, stroke
Nephrology	30	15 (50.0)	15 (50.0)	AKI
Neurology	100	70 (70.0)	30 (30.0)	Stroke, encephalopathy
Obgyn	25	20 (80.0)	5 (20.0)	Recurrent pregnancy losses, POF
Ophthalmology	105	83 (79.1)	22 (20.9)	Uveitis, optic neuritis
Orthopedics	28	28 (100)	0 (0)	Trigger finger, CTS, arthritis
Psychiatry	2	2 (100)	0 (0)	Encephalopathy, psychosis
Pulmonology	35	32 (91.4)	3 (8.6)	ILD, poorly controlled asthma, PHT
Rheumatology	25	23 (92.0)	2 (8.0)	Raynaud's phenomenon, arthritis
Surgery	95	94 (98.9)	1 (1.1)	CLI, vascular aneurysms
Trauma	3	3 (100)	0 (0)	ICA dissection, TBI

Abbreviations: AKI: Acute kidney injury, CLI: Critical limb ischemia, CTS: Carpal tunnel syndrome, IBD: Inflammatory bowel disease, ICA: Internal carotid artery, ILD: Interstitial lung disease, Obgyn: Obstetrics and gynecology, PHT: Pulmonary hypertension, POF: Premature ovarian failure, TBI: Traumatic brain injury.

Table 3. Details of ten patients with anti-neutrophil cytoplasmic antibody-associated vasculitis

Gender	Age	Ethnicity	Department	AAV type	MPO	PR3	Guideline indication
M	55	Mixed	Medicine	GPA	Neg	Neg	Yes
F	49	Mixed	Medicine	RLV	Pos	Neg	Yes
F	37	Mixed	Medicine	RLV	Pos	Neg	Yes
M	81	Caucasian	Medicine	RLV	Pos	Neg	Yes
F	71	Mixed	Medicine	RLV	Pos	Neg	Yes
M	39	Mixed	Medicine, Nephrology	RLV	Neg	Pos	Yes
F	58	African	Medicine, Nephrology	RLV	Neg	Neg	Yes
F	58	Mixed	Nephrology	RLV	Neg	Neg	Yes
F	29	Mixed	Nephrology	RLV	Neg	Neg	Yes
F	68	Mixed	Medicine	RLV	Neg	Neg	Yes

Abbreviations: AAV: Anti-neutrophil cytoplasmic antibody-associated vasculitis, F: Female, GPA: Polyangiitis, M: Male, MPO: Anti-myeloperoxidase antibody, Neg: Negative, Pos: Positive, PR3: Anti-proteinase 3 antibody, RLV: Renal limited vasculitis.

lower than what has been described elsewhere due to the high number of false positive tests observed in our setting. The sensitivity, specificity, PPV, and NPV were 50.0%, 95.1%, 35.7%, and 97.2%, respectively. The total estimated cost of ANCA tests throughout the year amounted to ZAR274,046, with ZAR17,490 spent on duplicate testing and ZAR208,275 on tests for non-indicated clinical conditions**.**

4. Discussion

Due to its multi-system heterogeneous presentation, insidious onset, and rarity, small vessel vasculitis poses a diagnostic challenge. As a result, the ANCA

Table 4. Positive anti-neutrophil cytoplasmic antibody results in patients with no evidence of anti-neutrophil cytoplasmic antibody-associated vasculitis

Gender	Age	Ethnicity	MPO	PR3	Guideline indication	Indication description	Clinical information
M	47	African	Pos	Neg	Yes	GN	HIV, AKI due to hypertension nephrosclerosis
F	48	African	Pos	Neg	Yes	GN	HIV, HIVAN
F	43	African	Pos	Neg	No	Hepatitis	HIV, confirmed DILI
F	33	Mixed	Neg	Pos	No	Hepatomegaly and lymphadenopathy	HIV, HIV seroconversion
M	47	Mixed	Neg	Pos	No	Cholangitis	HIV, syphilis, confirmed liver abscesses
F	44	African	Pos	Pos	Yes	Palpable purpura	HIV, biopsy- cutaneous lupus
M	35	African	Neg	Pos	Yes	GN	TB, AKI, and macroscopic haematuria
F	38	African	Pos	neg	No	AKI	HIV, disseminated TB
F	30	African	Pos	Neg	Yes	PN	HIV, TB
M	39	African	Pos	Neg	No	Valve heart disease	HIV, TB, epilepsy
F	57	Mixed	Neg	Pos	No	Seizures	HPT, syphilis
F	80	Mixed	Pos	Neg	No	ILD	RA, HPT, hyperthyroidism
M	38	Caucasian	Pos	Neg	No	Hepatitis	AIH
F	55	African	Pos	Neg	No	Unclear	SLE
M	67	Mixed	Pos	Neg	Yes	GN	HPT, biopsy- diffuse glomerulosclerosis
F	69	Mixed	Pos	Neg	Yes	GN	HPT, DM, asthma, biopsy-MCGN, and ATN
M	47	African	Pos	Neg	No	AKI	-
F	62	Mixed	Pos	Neg	Yes	PN	HPT, ILD
F	68	Mixed	Pos	Neg	No	Myelopathy	HPT
F	73	Mixed	Pos	Neg	No	ILD	HPT, DM
F	54	Mixed	Pos	Neg	No	Poorly controlled asthma	HPT, asthma
M	29	African	Pos	Pos	No	Uveitis	-
M	66	Mixed	Neg	Pos	Yes	Scleritis	HPT
M	41	Asian	Neg	Pos	No	Amblyopia	-
F	48	Mixed	Neg	Pos	No	Raynaud's phenomenon	Breast cancer in remission
F	38	African	Pos	Neg	No	POF	-

Abbreviations; AIH: Autoimmune hepatitis. AKI: Acute kidney injury, ATN: Acute tubular necrosis, DILI: Drug-induced liver injury, DM: Diabetes mellitus, F: Female, GN: Glomerulonephritis, HIV: Human immunodeficiency virus, HIVAN: HIV associated nephropathy, HPT: Hypertension, ILD: Interstitial lung disease, LN: Lupus nephritis, M: Male, MCGN: Mesangiocapillary glomerulonephritis, MPO: Anti-myeloperoxidase antibodies, Neg: Negative, PN: Peripheral neuropathy, POF: Premature ovarian failure, Pos: Positive, PR3: Anti-proteinase 3 antibodies, RA: Rheumatoid arthritis, SLE: Systemic lupus erythematosus, TB: Tuberculosis.

test plays an important role as a screening tool. However, our study revealed that only a quarter of ANCA tests conducted had indications that met the 1999 international consensus statement guidelines. This indiscriminate use of ANCA testing is costly and lowers the test sensitivity and PPV. Similar studies conducted elsewhere reported that 33.4% and 50.0% of ANCA tests were performed within guideline indications [9,10]. Implementing gating policies to restrict ANCA testing has proven

beneficial, reducing false-positive rates by up to 27.0% and increasing ANCA positivity by 11.8% without missing AAV cases [3,11].

False-positive ANCA tests have been well-described, and in the present study, they resulted in a lower PPV than what has been described elsewhere [10,12-14]. In our study, among the 26 patients who tested positive for ANCA but showed no evidence of AAV, chronic infection was noted in 42.3% of the cases. Elsewhere, chronic infections, including TB, malaria, leprosy, suppurative lung disease, infective endocarditis, hepatitis B and C, and HIV, have been noted to cause positive ANCA results [5,15]. In the pre-highly active antiretroviral therapy (HAART) era, Koderisch *et al*. described c-ANCA positivity in 24 out of 29 HIV-infected patients (83.0%) [16]. More recently, among HIV patients with a well-controlled viral load on HAART, 45.0% had at least one autoantibody present, especially ANA (33.0%) and ANCA (13.0%), without clinically relevant disease [17].

In the present study, 11.5% of false positive ANCA tests were observed in patients with autoimmune diseases, which are consistent with findings reported elsewhere, particularly among patients with chronic autoimmune hepatitis (70.0%), rheumatoid arthritis, and systemic lupus erythematosus (20.0%) [18]. Other causes of positive ANCA results, including certain medications (anti-thyroid drugs, propylthiouracil, levamisole-adulterated cocaine, minocycline and hydralazine), and inflammatory bowel disease, have been described but were not encountered in our study. The high rate of false positive results underscores the need to increase the pre-test probability by limiting ANCA testing to the 1999 international consensus statement guidelines.

Our study shows a very low rate of PR3 positivity: only nine patients in the false-positive group and one in the confirmed AAV group tested positive for PR3. We believe that this finding reflects the low rate of positive tests in African populations. Therefore, we plan to conduct more prospective studies to further investigate this phenomenon.

The AAV group was predominantly RLV, with half of these patients showing positive ANCA serology. Elsewhere, ANCA-negative RLV has been well-documented, highlighting the importance of tissue diagnosis as the gold standard when small vessel vasculitis is highly suspected [19]. The ANCA-negative group exhibits poorer renal outcomes and less extrarenal involvement.

Limitations of this study include the imprecision of retrospective record review where clinician notes may have inadequately documented the test indication, together with a large number of missing records. In addition, this audit was conducted in a tertiary hospital, and the results are not generalizable to other health-care platforms.

5. Conclusions

Our study shows indiscriminate ANCA testing, with 75.6% of tests done outside of guideline indications, and duplicate testing, with large cost implications. We also demonstrated false positive tests resulting in a lower PPV than described elsewhere. Implementing restrictive protocols for ANCA testing according to the 1999 testing guidelines, together with the training of clinicians, is likely to reduce unnecessary tests, resulting in significant cost savings and a reduction in inappropriate referrals to sub-specialists.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Author Contributions

Conceptualization: All authors
Investigation: All authors
Writing-original draft: All authors
Writing-review and editing: All authors

Ethics Approval and Consent to Participate

Ethics approval was obtained for this study from The Human Research Ethics Committee of the Faculty of Health Sciences, University of Cape

Town, approved the study (HREC reference number 443/2018).

Consent for Publication

Not applicable.

Availability of data

Data can be obtained from the corresponding author following formal request.

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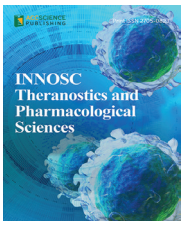

RESEARCH ARTICLE

Screening of Biological Activity of Selected Medicinal Orchids of Western Ghats, Karnataka, India

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Abstract

The present work was carried out to investigate the presence of bioactive compounds in selected medicinal orchids such as *Dendrobium jerdonianum*, *Dendrobium barbatulum*, *Eria mysorensis*, *Bulbophyllum neilgherrense,* and *Pholidota pallida.* The phytochemical, antibacterial, and antioxidant properties of the plants were evaluated using extracts obtained through chloroform, methanol, and aqueous extraction methods, respectively. The results of the phytochemical test showed the presence of diverse classes of secondary metabolites such as alkaloids, steroids, triterpenes, saponins, tannins, flavonoids, carbohydrates, resins, proteins, glycosides, and phenolics. The presence of these phytochemicals varied among different solvent extracts among the selected plants. Among the plant extracts tested, *D. Jerdonianum* and *P. pallida* offered significant $p \geq 0.05$ antibacterial properties against *Escherichia coli*, *Salmonella* typhi*, Bacillus subtilis*, *Shigella flexneri*, and *Pseudomonas aeruginosa* with a minimum inhibitory concentration (MIC) ranging from 0.312 to 2.5 mg/mL. Among the plant extracts tested, methanol extract of *Erythrina mysorensis* (29.38%)*, B. neilgherrense* (15.92%)*, P. pallida* (13.84%)*,* and *D. barbatulum* (13.10%) showed highest phenolic contents, followed by chloroform and aqueous extracts in all the plants. *E. mysoriensis* and *D. jerdonianum* exhibited highest antioxidant properties at 85.41% and 81.28% (with IC_{50} values of 36.49 and 43.72 µg/mL), respectively, while the positive control, gallic acid, displayed antioxidant properties of 94.61% (with an IC_{50} values of 30.49 µg/mL). These results warrant further studies on using these selected orchids for therapeutic and pharmaceutical applications.

Keywords: Antibacterial, Antioxidants, Orchids, Phytochemistry

1. Introduction

Medicinal plants such as herbs, shrubs, and woody plants have played a crucial role in the development of new therapeutic drugs, contributing to the improvement of human life for thousands of years. These biologically active therapeutic agents have served as a valuable source of new natural products for humans [1]. Orchidaceae is one of the biggest and most well-known families of flowering plants in the plant kingdom [2]. With approximately 779 genera and 22,500 species, it stands as the most ecologically and morphologically diverse member of angiosperms [3]. In India, the diversity of Orchidaceae members is represented by 1331 species across 186 genera, due to their adaptable nature. Orchids can be classified as epiphytic (growing on trees or shrubs), saprophytic (growing on dead and decaying matter), or terrestrials (growing on the ground). They are highly sensitive to habitat degradation and fragmentation [4,5].

The majority of orchid plants are highly medicinal and used to combat various ailments [6,7]. These plants are also used to extract various natural

products such as perfumes and medicines [8,9]. Approximately 60% of orchids are used every year for various medicinal purposes, and they are known to contain abundant chemical constituents such as alkaloids, flavonoids, and terpenoids [10,11]. One well-known example is vanillin, a flavoring agent obtained from *Vanillin planifolia* [12,13]. Vanillin is a phenolic aldehyde, commercially available, and extensively used in the food industry [14]. Purified compounds obtained from orchid extracts exhibit several bioactivities such as antimicrobial, antioxidant, anthelmintic, insecticidal, antiviral, analgesic, antipyretic, anti-allergic, wound healing, anti-aging, anti-cancer, and anti-inflammatory properties [15-17]. The Indian subcontinent, characterized by different climatic regimes, habitat conditions, and forest types, provides a favorable environment for a diverse group of orchid species [18,19]. In southern India, the Western Ghats is the main hub for many natural products, encompassing a wide range of forest areas, ranging from tropical wet evergreen forests to grasslands with rich flora, and enriched with several endangered flowering plant species [20]. The Western Ghats area of Karnataka, which includes Kodagu, Hassan, Chikmagalur, Shimoga, and Uttara Kannada, boasts a high diversity of orchids [20-22] **(Figure 1)**. Thus, in light of the medicinal importance of the orchids, certain epiphytic orchids, such as *Dendrobium jerdonianum* Wight, *Dendrobium barbatulum* Lindl, *Eria mysoriensis* Lindl, *B. neilgherrense* Wight, and *Pholidota pallida* Lindl (**Figure S1**) obtained from the Western Ghats region, were selected for the present study. The objective of this study is to isolate bioactive secondary metabolites and evaluate the phytochemical, antibacterial, and antioxidant properties of these orchid plants using various organic solvent extraction methods.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals were purchased from Sigma Aldrich and Hi-Media Lab. Pvt. Ltd. Bangalore, including DPPH (2,2-diphenyl-1-picrylhydrazyl [99%]), hexane (98%), chloroform (98%), methanol (99%), sterile distilled water and streptomycin, gallic acid (99%), nutrient agar, nutrient broth, Lieberman–Burchard (LB) reagent (98%), Folin–Ciocâlteu (FC) reagent (98%), phosphate buffer, and ferric chloride.

2.2. Plant materials collection

Fresh plant materials were collected from the Western Ghats of Karnataka, India (12° 25’37 N) and (75° 44’51 E) and identified using the Flora of the Presidency of Madras [23]. The specimens were submitted to the Department of Studies in Botany, University of Mysore (Voucher specimen no.: *D. jerdonianum* (UOMBOT20DJ05), *D. barbatulum* (UOMBOT20DB04), *E. mysoriensis* (UOMBOT20EM06), *B. neilgherrense* (UOMBOT20BN03), and *P. pallida* (UOMBOT20PP07).

2.3. Preparation of plant extracts

Collected plant materials (pseudobulbs/tubers) were thoroughly washed with running tap water, cut into small pieces, shade dried at room temperature, and made into a fine powder using a mixture grinder. Each powdered sample (100 g) was mixed in 250 mL of chloroform, methanol, and water, respectively, and left for 24 h in a rotary shaker at room temperature and then filtered. The filtrate was collected and subjected to flash evaporation to obtain the extracts. The extracts were stored at 4°C in an airtight glass bottle until further experiments [24].

2.4. Preliminary phytochemical screening

Each plant extract was subjected to qualitative preliminary phytochemical analysis to detect the presence or absence of various classes of secondary metabolites such as alkaloids, carbohydrates, flavonoids, glycosides, phenolics, proteins, resins, triterpenes, steroids, saponins, and tannins, using the method described by Harborne (1998) [25].

2.5. Antibacterial activity

2.5.1. Test pathogens

Bacterial strains, such as *Salmonella* typhi (MTCC733), *Shigella flexneri* (MTCC1457), *Pseudomonas aeruginosa* (MTCC2453), *Escherichia coli* (MTCC7410), and *Bacillus subtilis* (MTCC121), were procured from the Microbial Type of Culture Collection (MTCC) and Gene Bank, Chandigarh, India.

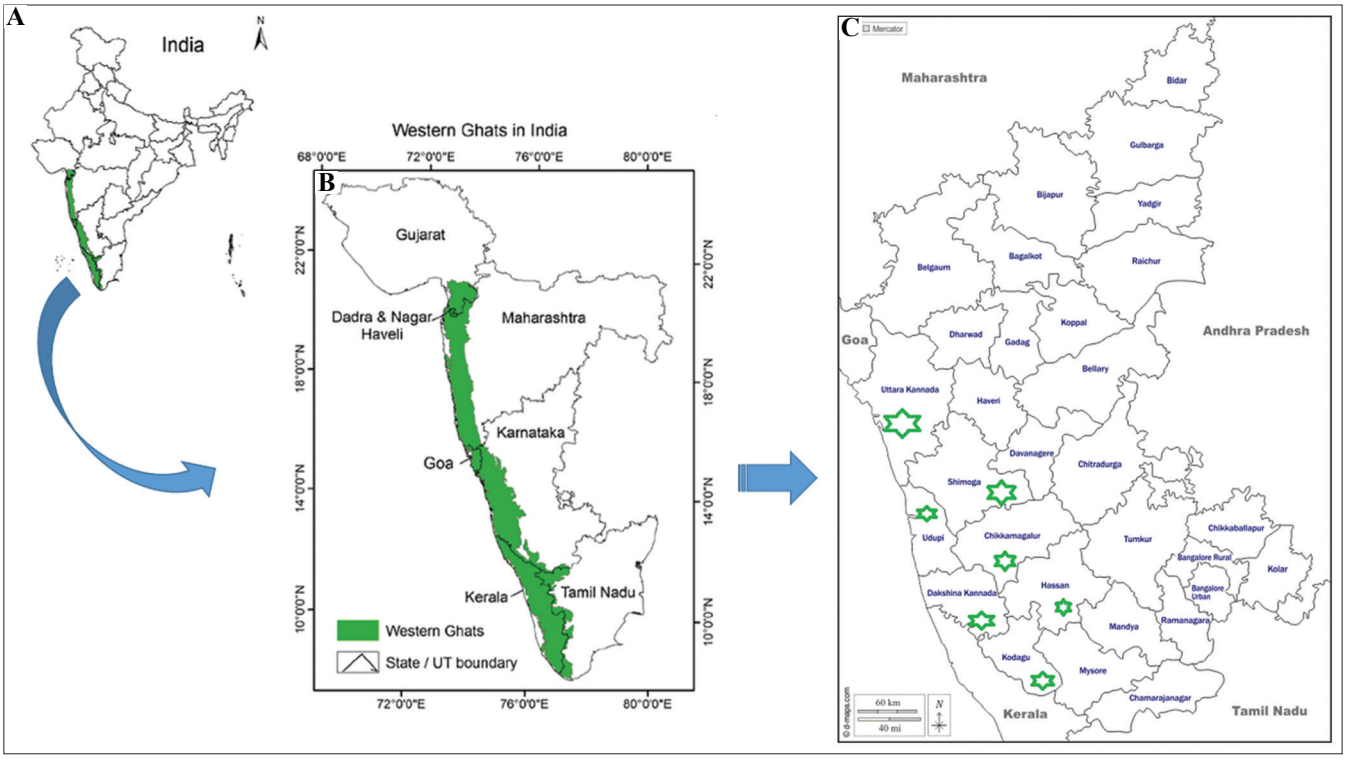

Figure 1. The map shows the diversity of orchids in the Western Ghats. (A and B) Western Ghats of India and their states. (C) Diversity of orchids in Karnataka districts.
Note: Green stars – indicate the areas inhabited by orchids.

2.5.2. Disk diffusion method

The antibacterial activity of the extract was tested using the disk diffusion method [26]. Briefly, 100 µL (1.5×10^8 CFU/mL) of the bacterial test sample (24-h-old) was spread uniformly on the surface of the Petri plates containing solidified nutrient agar media using a sterile glass spreader. The sterile disks (6 mm) were loaded with 50 µL (3.5 mg) and 75 µL (6.5 mg) of each plant extracts, respectively, from the stock (50 mg/mL) and placed equidistantly. The streptomycin (25 µg/mL) and respective solvents (25 µL) were used as positive and negative controls, respectively, and plates were incubated at 37±2°C for 24 h. After incubation, the zone of inhibition was measured, and the experiment was repeated in triplicates.

2.5.3. Minimum inhibitory concentration (MIC)

The MIC was determined using the micro-broth dilution method [27]. The extracts were prepared at a concentration of 50 mg/mL in respective solvents. In a 96-well plate, each well was dispensed with 100 µL of nutrient broth, followed by the addition of 100 µL stock solutions that were serially diluted (0.002–5 mg/mL). Subsequently, a 10 µL bacterial inoculum suspension was added to the mixture and incubated at 37±2°C for 24 h. Absorbance was measured without agitation at 620 nm using an ELISA plate reader (LabTech 4000). The MIC was confirmed by adding 10 µL of TTC (2, 3, 5-triphenyl tetrazolium chloride) per well at a concentration of 2 mg/mL and incubating the mixture for 30 min. The MIC was determined as the lowest concentration at which no color changes were observed. The experiments were performed in triplicates.

2.6. Antioxidant activity

The free radical scavenging activity (RSA) of each extract was evaluated using the DPPH assay, following the method described by Wahab *et al.* [28]. Each extract at varying concentrations (20, 40, 60, 80, and 100 µg/mL) was prepared from the stock of 1mg/mL and tested for antioxidant activity. Four milliliters (4 mg 100/mL in methanol) of DPPH solution was added to each test tube containing extract, and methanol only was used as blank. The reaction mixture was incubated for 20 min at room temperature in the dark. The absorbance was measured

at 517 nm after incubation using a spectrophotometer. The experiment was performed in triplicates. The percentage of RSA and the inhibitory concentration (IC_{50}) were calculated using the following formula:

$$\% \text{ Scavenging activity} = \frac{\text{Absorbance}(\text{control})\text{-Absorbance (sample)}}{\text{Absorbance (control)}} \times 100 \quad \text{(I)}$$

2.7. Total phenolic content

The presence of phenolic substances was measured using the FC reagent method, referring to gallic acid standard [29]. Each plant extract was tested at a concentration of 100 μg/mL. A combination of 0.5 mL of FC reagent and 1.0 mL of Na_2CO_3 (20%) was added to each test tube and incubated in the dark for 42 min. The absorbance was measured at 765 nm using a spectrophotometer (Thermo Fischer). The experiment was performed in triplicates. The absorbance of samples was plotted against the concentration and expressed in terms of gallic acid equivalence (μg GAE/g of extract).

2.8. Statistical analysis

Data from three replicates were averaged and analyzed for each experiment. Analysis of variance was performed using SPSS Inc. 16.0. To determine the significant effects of treatments, Tukey's HSD test was conducted to compare, and separate treatment means based on *F*-values ($p \leq 0.05$).

3. Results and discussion

3.1. Preliminary phytochemical screening

Plants with a greater number of phytoconstituents generally exhibit higher medicinal properties. The phytochemical analysis of tested plant extracts revealed the presence of various classes of secondary metabolites in chloroform, methanol, and aqueous extracts, and the results of the biochemical test showed the presence of various classes of phytoconstituents such as alkaloids, steroids, triterpenes, saponins, tannins, flavonoids, carbohydrates, resins, proteins, glycosides, and phenolics in the plant. The results are presented in **Table 1**.

Among the tested plants, *D. barbatulum* showed no alkaloids, while *B. neilgherrense* and *D. Jerdonianum* were positive for the majority of the test in all extracts. These results are consistent with findings reported by Nagananda *et al.* [30], which found alkaloids, flavonoids, phenols, phytosterols, and other compounds in the methanol extracts of *P. pallida.* The majority of the phytochemical tests yielded the highest number of positive results in methanol extracts, followed by aqueous and chloroform extracts in all the plants. The findings align with previous reports by Kumari *et al*. [31] on *B. neilgherrensis* and Maridass *et al*. on *D. barbatulum*, where the majority of the phytochemicals were present in methanolic and aqueous extracts [32]. The study results indicate that methanol is the most effective solvent for extracting secondary metabolites from orchid plants. Methanol has been found to dissolve the highest number of secondary metabolites. These findings are consistent with the previous research conducted by Esmaeili *et al*., who reported that methanol extracted the highest phytochemical content in *in vitro*-grown *Trifolium pratense* L. [33].

3.2. Antibacterial activity

The antibacterial activity of the crude extracts from selected orchids was evaluated at 50 mg/mL stock solution, and the results are presented in **Table 2**. The MIC values for each crude extract are shown in **Table S1**. Among the tested plant extracts, the methanolic extract of *P. pallida* effectively inhibited *E.coli* (18.64 mm), *P. aeruginosa* (15.36 mm), *S. typhi* (14.32 mm), *S. flexinari*(13.24 mm), and *B. subtilis* (13.34 mm) (**Figure 2**). The MIC values ranged 0.312–2.5 mg/mL. The methanol extract of *D. Jerdonianum* showed moderate antibacterial activity against all the tested bacteria, with inhibition zones of 9.54, 9.64, 10.24, 11.64, and 8.58 mm, respectively. The MIC values for *D. Jerdonianum* ranged from 0.625–2.5 mg/mL. No antibacterial activity was observed in the methanolic extracts of *D. barbatulum* and *Erythrina mysorensis*. The methanol extracts of *B. neilgherrense* only showed inhibition against *E.coli* (8.54 mm) and *S. flexinari* (8.12 mm), while chloroform and aqueous extracts of the other plants showed negative results. The results align with the previous reports by

Table 1. Preliminary phytochemical screening of selected orchids in various solvent extracts

Phytochemical tests	*D. jerdonianum*			*D. barbatulum*			*E. mysorensis*			*B. neilgherrense*			*P. pallida*		
	Chl	Meth	Aq	Cchl	Meth	Aq	Chl	Meth	Aq	Chl	Meth	Aq	Chl	Meth	Aq
Sterols															
Salkowski	-	-	-	-	+	-	-	-	-	+	+	-	-	+	-
LB test	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+
Triterpenes															
Salkowski	-	-	-	-	+	-	-	-	-	+	+	-	-	+	-
LB test	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+
Saponins															
Foam	+	-	-	+	-	-	+	-	+	+	-	-	+	-	+
Alkaloids															
Mayer's	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Dragondroff's	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-
Wagner's	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-
Hager's	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Tannins															
FeCl3	-	+	+	-	+	+	-	+	+	+	+	+	-	+	+
Gelatine	-	+	-	-	-	-	+	+	-	+	+	-	+	+	-
Flavonoids															
Shinado	+	-	+	+	+	+	+	+	+	+	-	-	+	-	-
FeCl3	-	+	+	-	+	+	-	+	+	+	+	-	+	+	+
Lead acetate	+	+	+	+	+	-	+	+	+	+	+	-	-	+	+
Carbohydrates															
Molisch's	+	+	+	-	+	+	-	+	+	+	+	-	+	+	+
Fehling's	+	+	+	-	-	-	+	-	-	+	-	-	+	+	-
Benedict's	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Resins															
Turbidity	+	-	+	+	-	-	+	-	-	+	-	-	+	-	-
Acetic anhydride	-	+	+	-	-	-	-	-	-	+	+	-	-	-	-
Proteins															
Biuret	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+
Ninhydrin	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+
Glycosides															
Killer-killiani	+	+	+	+	+	-	+	+	-	+	+	-	+	+	-
Phenolics															
Ferric chloride	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+

Notes: "+": presence;"-": absence; Aq: Aqueous; Chl: Chloroform; Meth: Methanol; *D. jerdonianum*: *Dendrobium jerdonianum*; *D. barbatulum: Dendrobium barbatulum*; *E. mysorensis: Erythrina mysorensis*; *B. neilgherrense: Bulbophyllum neilgherrense*; *P. pallida: Pholidota pallida*

Rashmi *et al*. on *P. pallida*, which demonstrated effective inhibition *B. subtilis*, *B. coagulans*, *S. typhi,* and *E. coli* using methanol extracts [34]. The results indicated that among the tested plant extracts, methanolic extract was found to possess significantly higher antimicrobial properties due to the presence of a higher number of secondary metabolites, as evidenced in the preliminary phytochemical screening test. Extracts with high potential secondary metabolites effectively

Table 2. Antibacterial activity of selected orchids in various solvent extracts

Plant and extract	Zone of inhibition measured in mm											
	E. coli		*P. aeruginosa*		*S. typhi*		*B. subtilis*		*S. flexneri*		Standard (25 µg)	Negative control
	50µL	75 µL	50 µL	75 µL	50 µL	75 µL	50 µL	75 µL	50 µL	75 µL		
D. jerdonianum												
Chl	-	-	-	-	-	-	-	-	-	-	17.20±0.25	-
Meth	8.12±0.25	9.54±0.54	-	8.64±0.54	9.64±0.98	10.24±0.87	9.22±1.24	11.64±0.54	8.58±0.98	8.24±0.26	15.12±0.25	-
Aq	-	-	-	-	-	-	-	-	-	-	17.23±0.63	-
D. barbatulum												
Chl	-	-	-	-	-	-	-	-	-	-	14.63±0.54	-
Meth	-	-	-	-	-	-	-	-	-	-	17.25±0.56	-
Aq	-	-	-	-	-	-	-	-	-	-	15.56±0.85	-
E. mysorensis												
Chl	-	-	-	-	-	-	-	-	-	-	11.12±0.64	-
Meth	-	-	-	-	-	-	-	-	-	-	12.54±0.32	-
Aq	-	-	-	-	-	-	-	-	-	-	10.44±0.87	-
B. neilgherrense												
Chl	-	-	-	-	-	-	-	-	-	-	13.12±0.25	-
Meth	8.54±0.87	8.54±0.24	-	-	-	-	-	-	-	8.12±0.39	19.56±0.74	-
Aq	-	-	-	-	-	-	-	-	-	-	13.26±0.26	-
P. pallida												
Chl	-	-	-	-	-	-	-	-	-	-	17.64±0.54	-
Meth	11.14±0.64	18.64±0.24	14.25±0.46	15.36±0.52	11.32±0.24	14.32±0.57	12.32±0.62	13.24±0.65	12.21±1.32	13.34±0.29	15.54±0.54	-
Aq	-	-	-	-	-	-	-	-	-	-	18.36±0.58	-

Note: Values are means of three independent replicates±SE. Notes: "-": absence; Aq: Aqueous; Chl: Chloroform; Meth: Methanol; negative control: Respective solvents; standard: Streptomycin; *E. coli: Escherichia coli*; *P. aeruginosa*: *Pseudomonas aeruginosa; S.* typhi: *Salmonella* typhi*; B. subtilis: Bacillus subtilis; S. flexneri: Shigella flexneri*; *D. jerdonianum*: *Dendrobium jerdonianum*; *D. barbatulum: Dendrobium barbatulum*; *E. mysorensis: Erythrina mysorensis*; *B. neilgherrense: Bulbophyllum neilgherrense*; *P. pallida: Pholidota pallida*

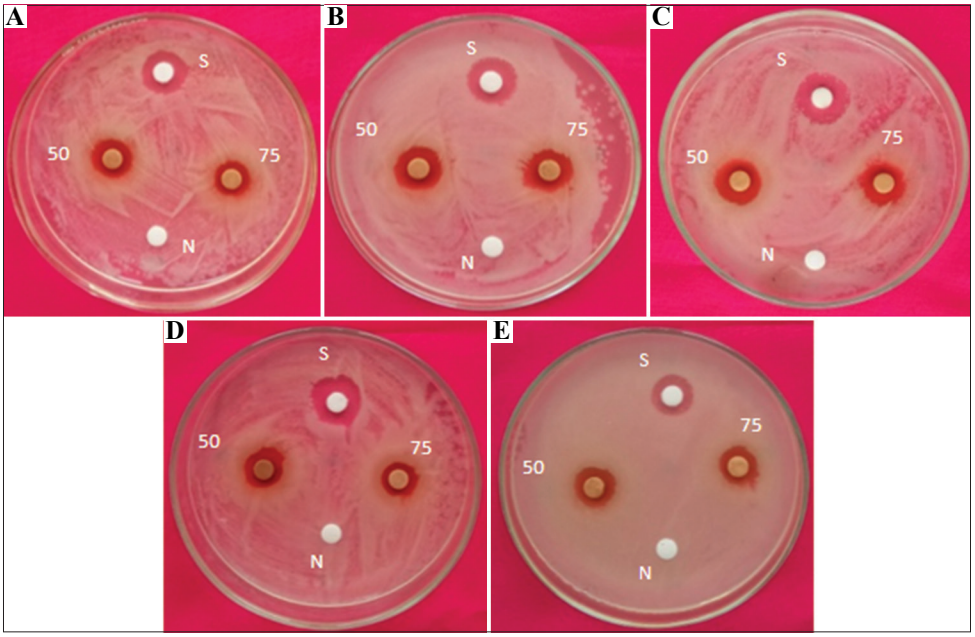

Figure 2. The antibacterial activity of the maximum zone of inhibition showed in methanolic extracts of *Dendrobium jerdonianum*. (A) *Salmonella typhi*, (B) *Shigella flexneri*, (C) *Pseudomonas aeruginosa*, (D) *Escherichia coli*, and (E) *Bacillus subtilis*.
Notes: N: Negative control; S: Standard.

inhibited the growth of bacteria, a finding in line with the report of Bhatnagar and Ghosal (2018), who demonstrated that methanolic extracts effectively inhibit *E. coli*, *S. aureus,* and *Acinetobacter* sp. It was also noticed that the zone of bacterial inhibition was also increased with plant extracts in a dose-dependent manner [35].

3.3. Antioxidant activity

The antioxidant activity of each plant extract was evaluated using the DPPH method. The percentage of RSA was measured, and the results are presented in **Table 3**. Among the tested plant extracts, aqueous extracts of *P. pallida, D. jerdonianum,* and *B. Neilgherrensis* exhibited antioxidants properties of 91%, 81%, and 74%, respectively, with inhibitory concentrations (IC_{50}) of 55.68, 43.72, and 54.84 µg/mL. The methanol and chloroform extracts of *D. barbatulum* and *E. mysorensis* showed significant RSA of 83% and 85% at higher concentrations, with IC_{50}values of 44.02 and 36.49 µg/mL, respectively. The standard gallic acid showed 94% inhibition at 100 µg/mL (IC_{50} = 30.49 µg/mL), and it was dose-dependent. Similar results on medicinal orchids have been reported by Chand *et al*., who screened various solvent extracts for antioxidant properties [36]. The results revealed that the aqueous extract showed excellent antioxidant activity, followed by the methanol and chloroform extracts. The assay also revealed a dose-dependent with RSA. Furthermore, Paudel *et al*. reported the antioxidant activity of *D. moniliforme*, wherein the ethanol extracts showed antioxidant properties of 94%, significantly higher than acetone and chloroform extracts [37].

3.4. Total phenolic content (TPC)

The total phenolic content was measured and quantified in terms of gallic acid equivalent. These plants are often associated with polyphenolic substances and are considered a good source of antioxidants. Orchid plants, in particular, are well-known for their own medicinal properties (Moretti *et al.*, 2013) [38]. The phenolic content was measured according to the Folin–Ciocâlteu method at 100 µg/mL, and the results are presented graphically in **Figure 3**. The assay revealed that all the extracts contained a considerable amount of polyphenols. The percentage of TPC was determined by referencing the plotted standard gallic acid curve and applying a linear regression coefficient with an R^2 value of 0.9986 **(FigureS2)**. Among the tested plant extracts, the methanol extracts of *E. mysorensis*, *B. neilgherrense*, *P. pallida*, and *D. barbatulum* showed the highest phenolic

Table 3. DPPH radical scavenging activity of selected orchids in various solvent extracts

Plants and extracts	Concentrations (µg/mL)					IC_{50} (µg/mL)
	20	40	60	80	100	
D. jerdonianum						
Chloroform	4.37±2.41^{n}	25.27±0.39^{n}	37.42±0.06^{k}	40.34±0.22^{j}	54.07±0.47^{k}	83.77
Methanol	23.93±0.82^{l}	39.85±0.19^{h}	47.75±2.13^{h}	56.86±0.13^{h}	62.21±1.42^{i}	68.30
Aqueous	38.91±0.81^{c}	47.26±1.61^{e}	57.22±0.65^{c}	68.40±0.26^{d}	81.28±0.97^{e}	43.72
D. barbatulum						
Chloroform	28.18±0.55^{j}	32.80±0.68^{k}	45.32±1.99^{i}	51.64±0.34^{i}	65.97±0.13^{h}	71.06
Methanol	32.07±0.76^{g}	51.76±1.85^{c}	62.21±0.42^{b}	67.92±2.24^{e}	83.83±0.61^{d}	44.02
Aqueous	31.59±1.74^{h}	51.76±0.85^{c}	62.21±0.42^{b}	70.83±0.61^{c}	59.65±0.78^{j}	46.14
E. mysorensis						
Chloroform	39.79±0.51^{b}	54.44±0.71^{b}	61.44±0.93^{b}	74.08±0.62^{b}	85.41±0.92^{c}	36.49
Methanol	33.90±0.36^{d}	50.18±2.26^{d}	56.74±0.62^{d}	68.28±0.75^{d}	75.69±0.66^{f}	42.47
Aqueous	29.76±0.14^{i}	34.38±0.39^{j}	39.85±0.19^{j}	40.94±0.75^{j}	61.60±0.89^{i}	84.76
B. neilgherrense						
Chloroform	23.57±0.23^{l}	27.46±0.51^{m}	29.16±0.16^{l}	33.29±0.83^{k}	38.88±2.14^{m}	47.79
Methanol	12.02±0.16^{m}	21.74±0.97^{o}	26.00±0.43^{m}	33.17±1.32^{k}	45.20±0.49^{l}	117.50
Aqueous	35.96±0.98^{d}	41.31±2.27^{g}	51.03±0.81^{f}	59.29±0.26^{f}	74.72±0.61^{f}	54.84
P. pallida						
Chloroform	33.04±0.82^{f}	38.03±1.59^{i}	44.10±0.93^{i}	57.71±0.67^{g}	68.40±0.26^{g}	63.87
Methanol	31.34±0.72^{h}	42.64±0.85^{f}	55.52±0.55^{e}	72.29±0.48^{c}	82.38±1.53^{e}	49.67
Aqueous	26.85±0.98^{k}	28.67±0.58^{l}	49.08±0.87^{g}	72.90±2.01^{c}	91.13±0.21^{b}	55.68
Standard						
Gallic acid	41.33±0.51^{a}	59.41±0.65^{a}	68.38±0.61^{a}	80.34±0.58^{a}	94.61±0.47^{a}	30.49

Values are the means of three independent replicates±SE. Means followed by the same letter (s) within the same column are not significantly different, according to Tukey's HSD.

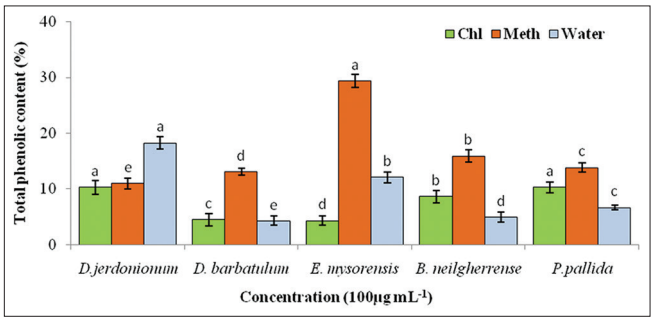

Figure 3. Total phenolic contents of selected orchid in various solvent extracts at the concentration of 100 µg/mL and expressed in gallic acid equivalence per gram of dry weight. Values are the means of three independent replicates (n = 3), and bars sharing the same letters are not significantly different ($p \leq 0.05$) according to Tukey's HSD test results.
Notes: Chl: Chloroform; Meth: Methanol.

contents of 29.38%, 15.92%, 13.84%, and 13.10%, respectively. The aqueous extracts *D. Jerdonianum* showed a phenolic content of 18.26%, followed by the chloroform extracts. The findings are in agreement with the studies conducted by Poudel *et al*. [39] on *D. amoenum* and Minh *et al*. [40] on *Phalaenopsis* sp., which reported significant phenolic and flavonoid contents in these orchid species, making them good source of antioxidants [40].

4. Conclusion

This study highlights the bioefficacy of selected medicinal orchids from the Western Ghats. The phytochemical analysis in various solvent extracts revealed the presence of secondary metabolites such as alkaloids, steroids, triterpenes, saponins,

tannins, flavonoids, carbohydrates, resins, proteins, glycosides, and phenolics. The diverse range of phytochemicals present in the tested extracts contributes to their potential antioxidant properties and their status as good sources of phenolic substances. Furthermore, among the various extracts tested, methanolic extracts of *D. Jerdonianum* and *P. pallida* showed significant inhibition against tested pathogens, indicating the potential of isolating the bioactive metabolites for antibacterial purposes. The overall bioefficacy of the selected orchids can be attributed to the presence of numerous groups of secondary metabolites in various solvent extracts. Therefore, the study paves the way for their better utilization as therapeutic agents in the near future.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Author contributions

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

The data generated and analyzed during this study are included in this article and provided some data as supplementary files. The data are available at the corresponding author's request.

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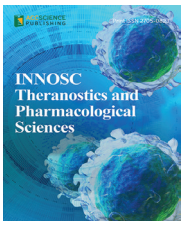

RESEARCH ARTICLE

Inhibitory Potential of Chitosan Derivatives against Severe Acute Respiratory Syndrome Coronavirus 2: An *In Silico* Prospective

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Abstract:

The present work was designed to investigate the antiviral potential of novel monomeric and oligomeric chitosan derivatives through *in silico* approaches. The goal was to identify potent broad-spectrum antiviral compounds as promising drug candidates against severe acute respiratory syndrome coronavirus 2 and understand their mode of action. Chitosan biopolymer and its derivatives were virtually screened against the spike glycoprotein and human angiotensin-converting enzyme 2 (ACE2) receptor of novel coronavirus-19. Hydroxypropyl trimethyl ammonium chloride chitosan (HTCC), a polymeric chitosan, has been reported to interact with the corona viral spike (S) protein and blocks its interaction with the ACE2 receptor. The enhancement of antiviral activity relies on better biocompatibility, structural correlations, variation in the degree of deacetylation, and molecular weight of modified chitosan derivatives. The chitosan derivatives constructively interact with viral S protein. Among the chitosan derivatives, N-carboxymethyl chitosan (NCMC) displayed efficient binding affinity. Comparing NCMC to mHTCC, monomeric chitosan, for their interaction with the S protein, receptor binding domain site, and ACE2 receptor, NCMC displayed better binding affinity of −7.9, −6.3, and −7.4 with binding energies of −6.2, −4.8, and −5.5 kcal/mol, respectively. Furthermore, through flexible docking, the interactions of the S protein with ACE2 receptor and ligand mHTCC-S protein complex and NCMC-S protein complex with ACE2 receptor were calculated, showing an efficient reduction of binding energy from −901.2 kJ/mol to −765.06 kJ/mol and −814.72 kJ/mol, respectively. This points to the decrease binding affinity of the viral S protein for the ACE2 receptor in the presence of NCMC/mHTCC. For the first time, the computational study envisages the antiviral efficiency of NCMC, mHTCC, and biocompatible chitosan derivatives as a preventive intervention against COVID-19.

Keywords: Chitosan, mHTCC, Severe acute respiratory syndrome coronavirus 2, S protein, ACE2 receptor, Molecular interaction

1. Introduction

Novel coronavirus-19 (nCoV-19) pandemic has emerged as a global health crisis, resulting in 18,055,630 deaths globally, yet a definitive cure remains elusive. The urgent need of the hour is to search for a safer and more potent drug with a high virucidal effect against nCoV-19 [1,2]. Therefore, the investigation of novel bioactive polymers for identification and cataloging becomes a fundamental necessity [3]. Chitosan, a poly [β-(l-4)-2-amino-2-deoxy-D-glucopyranose], is a profusely available

natural biopolymer with repeated chains of N-acetyl glucosamine and glucosamine. It possesses physical and biological functionality, is biodegradable, and demonstrates biocompatibility with various organs, tissues, and cells, obviated with cytotoxic and noxious effects [4-6]. The efficiency of chitosan in biomedical applications, such as drug and vaccine delivery, antimicrobial properties (bactericidal, virucidal, fungicidal, and anti-parasitic effects), wound healing, chitosan-guided bone regeneration, and the preparation of artificial skin, hydrogel, and bio lenses for eyes have revolutionized polymer science [5,7-11]. Chitosan's intermolecular and intramolecular hydrogen bonds contribute to its high crystallinity, making it almost insoluble in water and therefore limiting its applications to some extent. However, its adhesion capacity is amplified by the presence of several reactive groups, making it an excellent natural biopolymer. Moreover, chitosan's hydrophobicity and pH sensitivity enable heterogeneous biomedical applications. By reacting with various organic acids and their derivatives, chitosan can have aliphatic or aromatic acyl groups added to its chain [4,5]. Carboxylated chitosan, for instance, possesses higher water solubility and exhibits better thickening, heat preservation, film formation, flocculation, and kneading properties than chitosan alone. The hydrophilic positively charged quaternary ammonium group plays a crucial role in its properties. The higher degree of substitution (DS) in carboxyl chitosan improves water solubility, increases its charge and strength, and weakens hydrogen bonding. Carboxymethyl chitosan, an active compound in the biomedical and pharmaceutical fields, exhibits antibacterial properties that promote wound healing. In addition, it demonstrates lipid-lowering, anti-arteriosclerosis, antiviral, anti-tumor, anti-coagulation, and hypoglycemic effects [12,13].

In recent years, the most commonly used quaternary ammonium salts are 2,3-epoxypropyl trimethyl ammonium chloride and 3-chloro-2-hydroxypropyl trimethyl ammonium chloride and N, N, N- trimethyl ammonium chloride [6,14-16]. Quaternary ammonium chitosan salt has demonstrated superior antibacterial, antiviral, biocompatible, biodegradable, non-toxic, mucoadhesive, and anti-inflammatory properties, rendering them ideal filler fibers in dressing materials. As a mucoadhesive biopolymer, chitosan nanoparticles effectively encapsulate both hydrophilic and lipophilic chemotherapeutic drugs, significantly improving the pharmacokinetic profile of the drug by increasing its solubility and stability in the biological system. Research has shown that hydroxypropyl trimethyl ammonium chloride chitosan (HTCC), a chitosan polymer, interacts with the coronaviral spike (S) protein, effectively blocking its interaction with cellular receptors, thereby preventing the entry of the virus into susceptible cells. This interaction highlights the potential of HTCC as a potent broad-spectrum antiviral drug, effectively hampering the replication of severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2) and MERS CoV in VeroE6/human aerial epithelium (HAE)/Huh-7 cell cultures, suggesting HTCC's inhibitory activity against the viral spike protein and its entry receptor interaction [17-19]. However, the exact mechanism of action for these quaternary ammonium chitosan remains unclear and their antiviral mode of action against other viruses has yet to be elucidated [18].

The S protein of the virus interacts with the host cell membrane receptor called angiotensin-converting enzyme 2 (ACE2), a monomeric membrane-associated receptor on human cells, enabling SARS-CoV-2 to enter host cells. According to Jena *et al.*, 2021 [2], the receptor binding domain (RBD) of the S-glycoprotein of SARS-CoV-2 binds ACE2. This RBD region is identical to that of SARS-CoV. Targeting the RBD region of the spike protein could potentially impede viral attachment to the ACE2 receptor and prevent viral entry into host cells, making it a promising target for viral infection prevention [17]. While recent research has proposed various natural products for developing or repurposing anti-SARS-CoV2 drugs that target the spike glycoprotein, to the best of our knowledge, none have explored the potential of chitosan and its derivatives for this purpose [18,19].

In a recently published research [16-18], several chitosan derivatives, namely, N-octadecanoyl-N-3-carboxy propionyl chitosan, palmitoyl-trimethyl-chitosan, N-carboxymethyl chitosan (NCMC), N,O-carboxymethyl chitosan, and carboxy ethyl chitosan, demonstrated effective actions against viral S protein and its

interaction with the cell receptor. Molecular docking and binding affinity studies revealed the potential of these chitosan derivatives as antiviral agents, particularly in their interaction with the S protein of SARS-CoV-2 **(Figure 1)**. Notably, hydroxypropyl trimethyl ammonium chloride chitosan (mHTCC), N,O-carboxymethyl chitosan, glycerol chitosan, methyl methacrylate chitosan, monomeric chitosan, NCMC, carboxy ethyl chitosan, N-octadecanoyl-N-3-carboxy propionyl chitosan, and palmitoyl-trimethyl-chitosan displayed binding affinities (ΔGb) of −6.2, −5.9, −4.9, −5.3, −7.9, −7.2, −6.4, and −6.9 kcal/mol, respectively. Among these, NCMC and mHTCC exhibited the highest binding affinities toward S protein as a polymer and monomer, respectively. Therefore, mHTCC and NCMC were selected for further analysis of their protein-protein interactions with the S protein-ACE2 complex.

Utilizing computational methods for this study not only reduced biological waste but also saved research time and costs. Therefore, the use of computational approaches allowed us to explore natural compound-based therapeutic interventions for COVID-19. The results also demonstrated the strong binding affinity of NCMC and mHTCC for both the S protein and the RBD site of the S protein.

2. Materials and methods

2.1. Selection of monomeric and oligomeric subunits of chitosan derivatives

The monomeric and oligomeric subunits of chitosan derivatives, which exhibit improved water solubility, pH sensitivity, stability, biocompatibility, and biodegradability resulting from alkylation, carboxylation, acylation, and quaternization, were selected for interaction studies with the S protein of SARS-CoV-2. The decision to choose these subunits was based on their reported antiviral spectrum and inhibitory activity against highly pathogenic human coronaviruses, as cited in **Table 1**. The monomeric and oligomeric subunits include mHTCC, N,O-carboxymethyl chitosan, glycerol chitosan, methyl methacrylate chitosan, monomeric chitosan, NCMC, carboxy ethyl chitosan, N-octadecanoyl-N-3-carboxy propionyl chitosan, and palmitoyl-trimethyl-chitosan.

2.2. Construction of 3D structures of chitosan derivatives

The 3D structures of chitosan and its modified derivatives were established using their canonical smiles strings retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Conversion

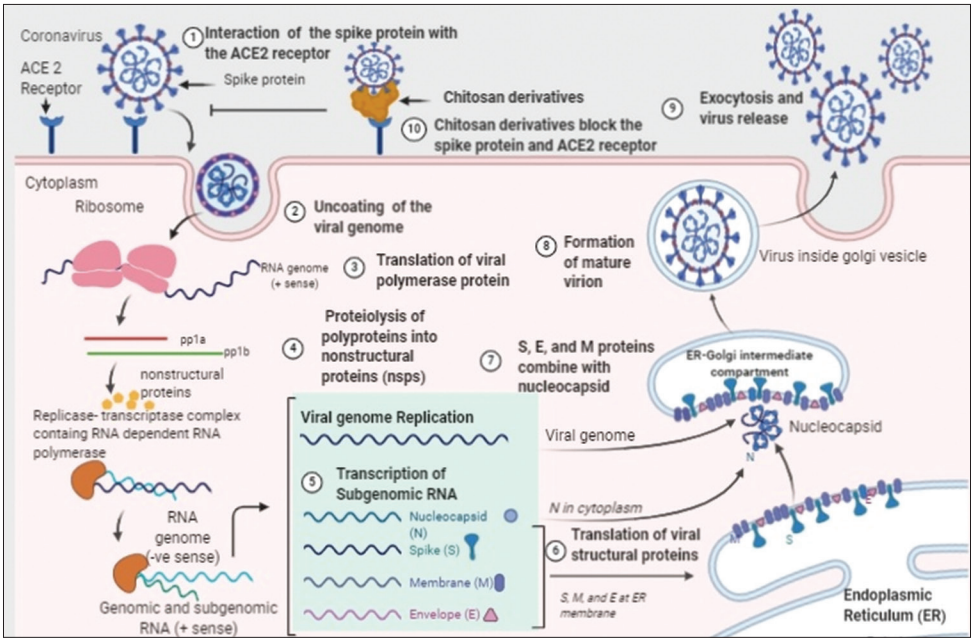

Figure 1. The attachment, entry, and multiplication of the severe acute respiratory syndrome coronavirus 2 in the host cell and its transport to the outer surface of the cell. Figure is generated using BioRender software.

Table 1. Type of modification, solubility parameters, molecular mass, antiviral spectrum, and mechanism of action of the selected chitosan derivatives

No.	Chitosan derivatives	Type of chitosan modification (molecular mass [g/mol])	Advantages of the polymer	Antiviral spectrum	Mode of action	References
1	Hydroxypropyl trimethyl ammonium chloride chitosan (mHTCC)	Quaternary ammonium addition (342.8)	Increases solubility, enhances mucoadhesion, prolongs the resistance, and increases pH sensitivity.	Hepatitis virus C, human coronaviruses (HCoVs), human immune deficiency virus-1 (HIV-1)	Inhibits the viral spike protein and host cell ACE2 receptor interaction and blocks viral propagation.	[17,18,37]
2	N,O-carboxymethyl chitosan	Methylation (291.2)	Enhances solubility in polar solvents with increasing biocompatibility.	HIV-1, Friend murine leukemia helper virus (F-MuLV), HSV	Inhibits the virus coat protein and host cell receptor interaction.	[20,30]
3	Glycerol chitosan	Glycerolyation (279.3)	Enhances penetrability and increases mucoadhesion.	Rotavirus, norovirus, adenovirus	Blocks viral replication.	[38]
4	Methyl methacrylate chitosan	Ammonium addition and alkylation (291.3)	Increases drug sustainability, slows down drug release, and increases penetrability.	Adenovirus and chicken pox and smallpox viruses	Inhibits viral protein synthesis.	[30]
5	Monomeric chitosan	No modification (180.18)	Enhances biocompatibility and biodegradability but is less soluble	Newcastle virus, influenza virus	Causes viral lysis.	[5]
7	NCMC	Methylation (559.5)	Enhances solubility in polar solvents with increasing biocompatibility	HIV-1, F-MuLV, HSV	Inhibits the virus coat protein and host cell receptor interaction.	[12]
8	Carboxy ethyl chitosan	Alkylation (573.5)	Increases solubility and stability	HIV-1, HSV, influenza virus	Inhibits the virus coat protein and host cell receptor interaction.	[20,30]
9	N-octadecanoyl-N-3-carboxy propionyl chitosan	Carboxylation and alkylation (868)	Increases solubility and enhances immunostimulatory activity and mucoadhesion.	Human noroviruses, murine viruses, feline caliciviruses	Interrupts the virus replication as well as interaction with the host.	[30]
10	Palmitoyl-trimethyl-chitosan	Methylation and palmitoylation (782)	Enhances access to the cell membrane and stabilizes the drug sustainability.	HSV, influenza virus, HIV-1	Inhibits viral polymerases and multiplication inside the host cell.	[31]

Abbreviations: HSV: Herpes simplex virus; ACE2: Angiotensin-converting enzyme 2.

into 3D structures was done using CHIMERA 1.11.2. Subsequently, these structures were used to evaluate their interactions with the spike protein of SARS-CoV-2 and the ACE-2 receptor [20].

2.3. Structure and sequence analysis of S protein and ACE- 2 receptor

The cryogenic electron microscopic structure of the S protein of SARS-CoV-2 (Protein Data Bank [PDB] ID: 6vsb) with a resolution of 3.46 Å and the X-ray diffraction structure of the ACE-2 receptor (PDB ID: 1r42) with a resolution of 2.2 Å were retrieved from the PDB. The FASTA sequences of the other reported human coronaviruses (HCoV-229E, MERS-CoV, HCoV-NL63, and SARS-CoV) were obtained from the PDB database. These sequences were then used for multiple sequence alignment to identify the similarity of the amino acid sequences of the three chains of the spike protein.

2.4. Phylogenetic analysis

The FASTA sequence of the S-protein was retrieved from the PDB database and evolutionary analysis of genetic distance and diversity was conducted using MEGA-X. Phylogenetic analysis was performed using the Substitution Model Jones-Taylor-Thornton (JTT) [21], and standard error estimates were obtained through a bootstrap procedure (1000 replicates). The phylogenetic tree was then constructed using the maximum likelihood statistical method.

2.5. Molecular docking

The evaluation of the free binding energy of energy-minimized chitosan subunits and their monomeric and oligomeric derivatives with the S protein and ACE2 receptor was conducted using the molecular docking program AutoDock Tools 1.5.6. Molecular docking studies were also performed to assess the binding affinity of chitosan derivatives, namely, mHTCC and NCMC, with the RBD of the S protein. The protein binding affinity of S protein, RBD, and ACE2 receptor with chitosan monomeric and oligomeric derivatives was examined using AutoDock Vina1.1.2. Various parameters, such as binding affinity, receptors interacting atom, receptor pocket atom, receptor-ligand interaction site, atomic contact energy (ACE), and side amino acid residues, were studied to identify the binding sites of the S protein and ACE2 receptor [22]. The results of the docking studies were visualized and analyzed using Discovery Studio 2017 R2 Client [23-25].

2.6. Protein-protein interaction

Protein-protein interaction studies were carried out using flexible docking to investigate the interactions of specific protein complexes [1,2]. First, the interactions between the S protein with mHTCC and NCMC were examined using molecular docking. Subsequently, the resulting S protein-mHTCC and S protein-NCMC complexes were studied in their interaction with the ACE2 receptor. To assess the binding affinity of the S protein for the ACE2 receptor in the presence of mHTCC and NCMC similarly, the interaction between the S protein and ACE2 receptor without mHTCC and NCMC was also analyzed [26,27]. For this analysis, the clusPro 2.0 software (https://cluspro.bu.edu/publications.php) was employed, utilizing the fast Fourier transform (FFT) docking methods. The selections of the filtered conformations were based on the assessment of empirical free energy. Both the lowest de-solvation and electrostatic energies were taken into account for the evaluation of free energy. Piper being a FFT-based rigid docking tool, serves the clusPro clustering program for measure the native sites by providing 1000 low energy outcomes [28]. The native site is assumed to possess a wide range of free energies to yield a great number of results. Initially, a sample of approximately 10^9 positions of the ligand with respect to the receptor was taken. From this set, only the top 10^3 positions were selected for further analysis, considering all relative ligand positions corresponding to the receptor.

3. Results

3.1. Phylogenetic analysis

The S protein of SARS-CoV-2 showed the highest sequence identity (73.9090%) with SARS-CoV, while its lowest amino acid sequence similarity was observed with HCoV-229E (10.6077%). In addition, the S protein shares 22.9037% sequence identity with MERS-CoV and 18.5559% with HcoV-NL63. Phylogenetic tree analysis of SARS-CoV-2 and SARS-CoV reveals their close relationship, as they share the same operational taxonomic unit (out) **(Figure S1)**.

3.2. Molecular docking

3.2.1. Interaction of different monomeric subunits of chitosan with the S protein

The binding modes of monomeric units of chitosan and its derivative with the S protein were investigated using AutodockVina1.1.2. The free binding energies (ΔGb) of the S protein with energy-minimized chitosan monomeric and polymeric units, as well as its derivative, namely, mHTCC, N,O-carboxymethyl chitosan, glycerol chitosan, methyl methacrylate chitosan, monomeric chitosan, NCMC, carboxy ethyl chitosan, N-octadecanoyl-N-3-carboxy propionyl chitosan, and palmitoyl-trimethyl-chitosan, were calculated as −6.2, −5.9, −4.9, −5.3, −7.9, −7.2, −6.4, and −6.9 kcal/mol, respectively. Further information on the type of modification, solubility parameters, molecular mass, antiviral spectrum, and mechanism of action of the selected chitosan derivatives can be found in **Figures 2**, **3**, and **S2**–**S7**. **Table 2** provides a brief elaboration on the binding energy, interacting amino acids of the S protein, and the type of interaction with the selected chitosan derivatives. The 2D interaction between the S protein and the selected chitosan derivatives is provided in **Figures 2**, **3**, and **S2**–**S7**.

3.2.2. Interaction studies of mHTCC with the S protein

The mHTCC unit exhibits specific interaction with the S protein, particularly at the RBD site of the S protein and ACE2 receptor, with binding affinities recorded as −6.2, −4.8 and −5.5 kcal/mol, respectively. The present investigation elucidates that HTCC directly binds with certain amino acids of the S protein, including Glu1017, Ala1016, Ile1013, Thr961, Gln762, Ala958, Gln954, Arg1019, Arg1014, Gln954, Gln1010, Gln954, Arg765, and Leu1012, and those presented in the RBD, such as Ser438, Asp442, Phe342, Ala344, Asn440, Asn343, Asn437, Thr345, Trp436, Leu441, and Leu441. In addition, interactions with the ACE2 receptor involve Lys562, Tyr196, Gln102, and Ala99 (data on the interactions with the ACE2 receptor are not provided). These interactions primarily involve conventional hydrogen bonds, carbon-hydrogen bonds, unfavorable positive-positive interactions, alkyl bonds, pi-sigma bonds, pi-pi T-shaped interactions, van der Waals interactions, and unfavorable donor-donor interactions. **Figure 2** illustrates the 2D interaction of mHTCC with the S protein.

3.2.3. Interaction studies of NCMC with the S protein

The binding affinity exhibited by NCMC toward the S protein was found to be the highest, followed by carboxy ethyl chitosan, with binding energies of −7.9 and −7.2 kcal/mol, respectively. Nevertheless, the binding affinity of NCMC with the S protein was even higher than that exhibited by mHTCC, involving the participating amino acids, namely, Ala1016, Ala1016, Glu1017, Glu1017, Glu1017, Arg1019, Arg1019, Ala1020, Ala1020, Ser1021, Asn1023, Asn1023, Leu1024, Leu1024, Thr1027, Thr1027, Glu1031, Phe1042, Phe1042, Ala1016, Ala1020, Asn1023, Leu1024, Thr1027, Arg1039, Arg1039, Arg1039, and Arg1039. Further investigation into the binding energy of NCMC involved its interaction with the RBD of the S protein and ACE2 receptor. The binding affinity of NCMC for the RBD of the S protein was found to be -6.3 kcal/mol, involving the participating amino acids, such as Asn343, Arg509, Thr345, Ala344, Leu441, Trp436, Asp442, Ser438, Ser373, Ala372, Ser375, Asn437, Asn440, Ser375, and Phe374. Similarly, NCMC binding to ACE2 receptor exhibited binding energy of −7.4 kcal/mol, with primary participating amino acids including Asn397, Ala396, Arg514, Glu398, Asp206, Gln102, Ser511, Tyr510, Trp203, Tyr199, Gly205, Tyr202, Tyr196, Glu208, Gln98, Leu95, Lys562, and Ala396. These interactions involved conventional hydrogen bonds, carbon-hydrogen bonds, unfavorable positive-positive interactions, alkyl bonds, pi-alkyl bonds, pi-sigma bonds, alternative charges, salt bridges, van der Waals interactions, and unfavorable donor-donor interactions (data not provided). **Figure 3** illustrates the 2D interaction of NCMC with the S protein, the RBD of the S protein, and the ACE2 receptor, as well as participating amino acids at the RBD and ACE2 receptor, and the binding of NCMC with the S protein.

3.3. Protein-protein interaction

The best ten docking models with different free energies were obtained from the ClusPro web server, using the total root mean square deviation value as the grouping criteria [29]. From these models, we

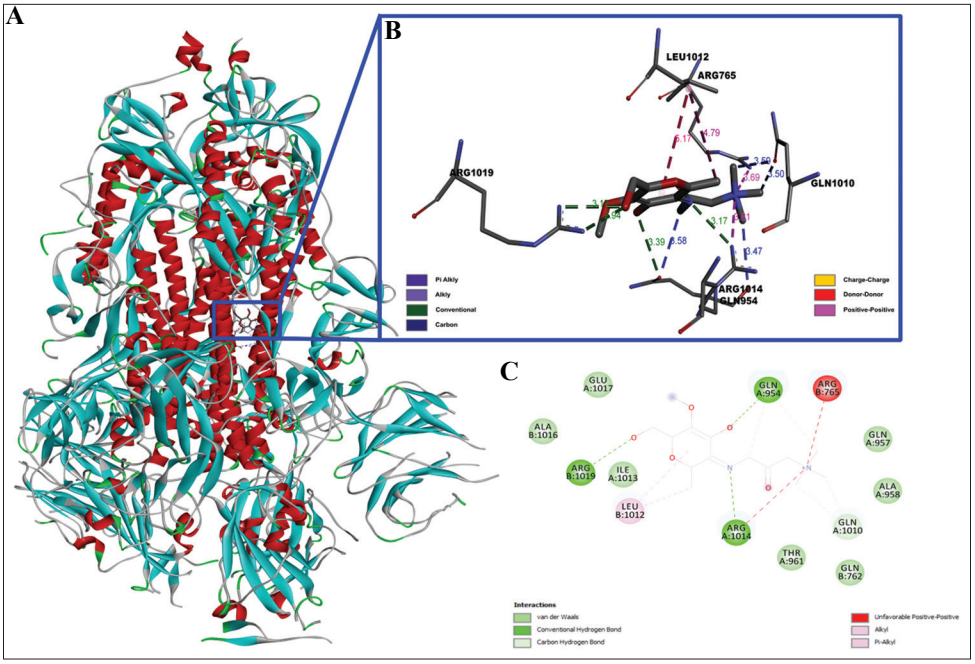

Figure 2. (A) Molecular docking of mHTCC with the S protein. (B) Brief 3D representation of mHTCC and S protein interaction including bond types and length. (C) 2D representation of mHTCC and S protein with participating amino acids and types of bonds.

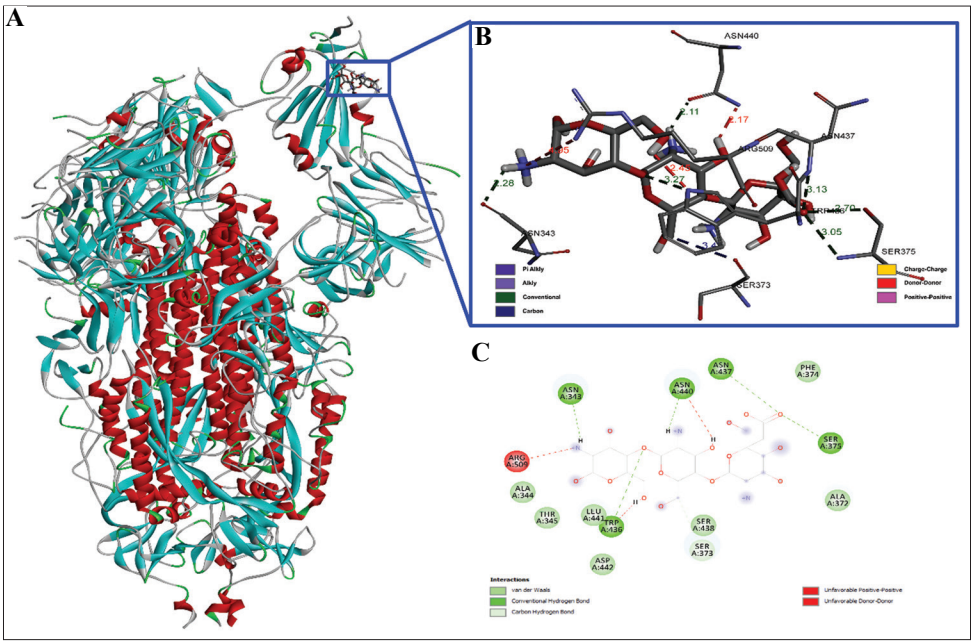

Figure 3. (A) Molecular docking of N-carboxymethyl chitosan (NCMC) with the S protein. (B) Brief 3D representation of NCMC and S protein interaction, including bond types and length. (C) 2D representation of NCMC and S protein with participating amino acids and types of bonds.

selected and analyzed five ClusPro docking models based on the probability of interaction between the S protein and derivatives of chitosan and the predicted binding sites of the ACE2 receptor, as well as the lowest binding energy observed during these interactions. The average binding energy for all five binding positions involved in S protein-ACE2 interaction is −901.2 kJ/mol. Nevertheless, in the presence of mHTCC and NCMC, the average binding energy for S protein-ACE2 interaction decreased to −765.06 kJ/mol and −814.72 kJ/mol, respectively (**Table 3**). These findings indicate that the free energy

Table 2. 2D representation of the molecular docking of the chitosan and its derivatives explaining the type of interaction and amino acids involved with detailed insight into the type of chitosan modification, advantages, its virucidal spectrum, and mechanism of action

No.	Chitosan derivatives	Binding affinity (kcal/mol)	Types of interaction	Interacting AA name; chain name; AA number
1	Hydroxypropyl trimethyl ammonium chloride chitosan	−6.2	van der Waals	Glu A:1017, Ala B:1016, Ile A:1013, Thr A:961, Gln B:762, Ala A:958, Gln A:954
			Conventional hydrogen bond	Arg B:1019, Arg A:1014, Gln A:954
			Carbon-hydrogen bond	Gln A:1010, Gln A:954
			Unfavorable positive-positive	Arg B:765
			Alkyl	Leu B:1012
2	N,O-carboxymethyl chitosan	−5.9	van der Waals	Thr C:549, Thr C:572, Ile C:587, Asp A:745, Met A:740, Tyr A:741, Cys A:743, Asn A:856, Phe C:541, Leu A:977, Leu A:966, Asn A:978, Thr C:572, Val A:976
			Conventional hydrogen bond	Arg A:1000, Ile A:742, Thr C:573
			Unfavorable donor-donor	Arg A:1000
			Carbon-hydrogen bond	Asp C:571
3	Glycerol chitosan	−4.9	van der Waals	Leu A:977, Arg A:100, Ile A:742, Val A:976, Leu A:966, Thr C:572, Asp C:571, Pro C:589, Thr C:549, Asn A:978
			Conventional hydrogen bond	Cys A:743, Thr C:573
			Carbon-hydrogen bond	Tyr A:741, Met A:740
			Salt bridge	Asp A:745
4	Monomeric chitosan	−5.3	van der Waals	Lys C:1028, Leu C:1024, Thr A:1027
			Conventional hydrogen bond	Glu A:780, Gln A:784, Ala A:1026, Ser A:1030, Phe C:1042
			Alternative charge	Asp C:1041, Glu C:725
5	NCMC	−7.9	van der Waals	Ala A:1016, Ala B:1016, Glu A:1017, Glu B:1017, Glu C:1017, Arg B:1019, Arg C:1019, Ala A:1020, Ala B:1020, Ser C:1021, Asn A:1023, Asn C:1023, Leu B:1024, Leu C:1024, Thr B:1027, Thr C:1027, Glu B:1031, Phe A:1042, Phe B:1042
			Conventional hydrogen bond	Ala C:1016, Ala C:1020, Asn B:1023, Leu A:1024, Thr A:1027, Arg B:1039, Arg C:1039, Arg A:1039
			Unfavorable donor-donor	Arg A:1039
6	Carboxy ethyl chitosan	−7.2	van der Waals	Gly A:757, Ser A:758, Cys A:760, Leu A:754, Cys A:738, Leu A:754, Thr A:739, Ser A:750, Asn A:764, Thr A:768, Leu C:303
			Alternative charge	Asp A:737
			Conventional hydrogen bond	Gln C:314, Thr A:761, Thr C:315, Thr C:302
			Unfavorable positive-positive	Arg A:765, Lys C:304
			Unfavorable donor-donor	Arg A:765, Lys C:304

(Cont'd...)

Table 2. *(Continued)*

No.	Chitosan derivatives	Binding affinity (kcal/mol)	Types of interaction	Interacting AA name; chain name; AA number
7	N-octadecanoyl-N-3-carboxy propionyl chitosan	−6.4	van der Waals	Pro A:665, Val B:772, Thr B:768, Asn B:764, Tyr A:913, Gln A:914, Ala B:766, Gln A:1010, Gln A:954, Arg A:1014, Gln A:957, Gly B:769, Leu B:1012, Gln B:762, Ala A:958, Thr B:761, Leu A:908
			Conventional hydrogen bond	Thr A:902, Ile :912
			Unfavorable positive-positive	Arg B:765
8	Palmitoyl-trimethyl-chitosan	−6.9	van der Waals	Gly A:667, Ile A:666, Gln A:613, Lys B:733, Ile A:312, Val B:772, Thr B:768, Tyr A:313, Leu A:303, Asn A:953, Ile B:770, Leu B:1012, Gly B:769, Gln A:954, Gln A:957
			Conventional hydrogen bond	Asp A:950, Arg B:765, Ala B:766
			Carbon-hydrogen bond	Asp A:950
			Alternative charge	Glu B:773
			Unfavorable positive-positive	Arg A:1014, Arg B:1019

Abbreviation: NCMC: N-carboxymethyl chitosan.

Table 3. Protein-protein interactions of S protein with ACE2 receptor, S protein-mHTCC complex with ACE2 receptor, and S protein-NCMC complex with ACE-2 receptor

Macromolecule	Binding positions	Lowest energy (kJ/mol)	Average lowest energy (kJ/mol)
S protein-ACE2	Red	−928.9	−901.2
S protein-ACE2	Green	−923	
S protein-ACE2	Blue	−902.4	
S protein-ACE2	Violet	−853.3	
S protein-ACE2	Light blue	−898.4	
S protein with mHTCC-ACE2	Red	−785.4	−765.06
S protein with mHTCC-ACE2	Green	−783.7	
S protein with mHTCC-ACE2	Blue	−750.4	
S protein with mHTCC-ACE2	Violet	−756.3	
S protein with mHTCC-ACE2	Light Blue	−749.5	
S protein with NCMC-ACE2	Red	−863.5	−814.72
S protein with NCMC-ACE2	Green	−850.2	
S protein with NCMC-ACE2	Blue	−741.1	
S protein with NCMC-ACE2	Violet	−797.3	
S protein with NCMC-ACE2	Light Blue	−821.5	

Abbreviations: NCMC: N-carboxymethyl chitosan; ACE2: Angiotensin-converting enzyme 2; HTCC: Hydroxypropyl trimethyl ammonium chloride chitosan.

ΔGb is reduced due to the binding of mHTCC to the S protein, as depicted in **(Figure 4** and **Figure S8)**. This finding complies with the study by Milewska *et al.* [17], indicating that both polymeric and monomeric HTCC reduce the affinity of the spike protein for the host cell ACE2 receptor. As a result, the virus replication and multiplication inside the host tissue are significantly reduced.

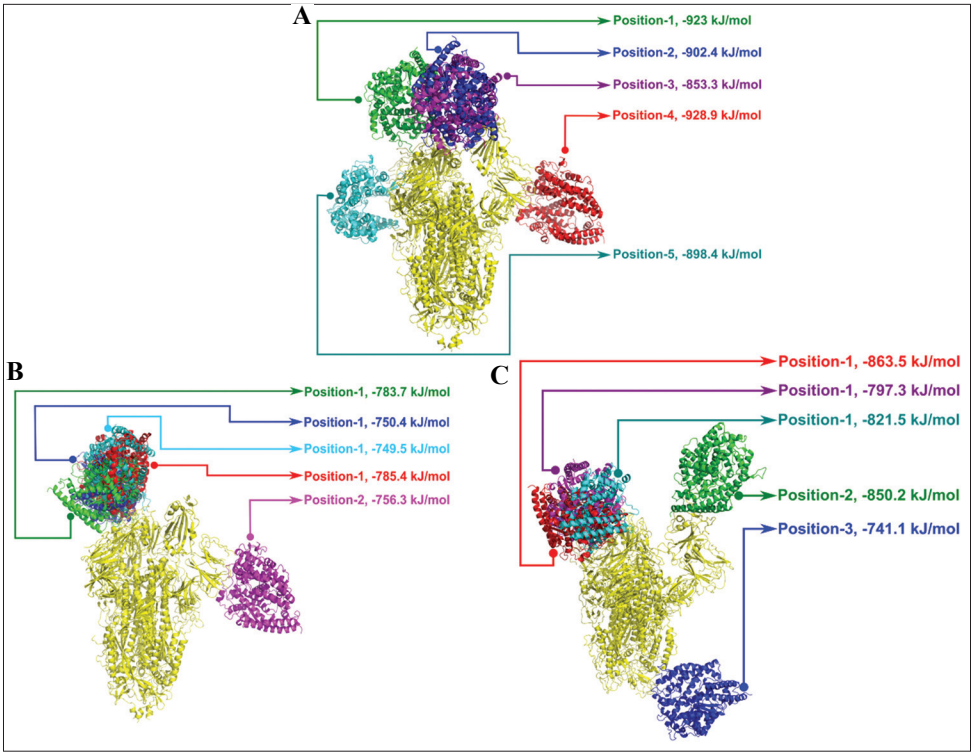

Figure 4. Protein-protein interaction of (A) angiotensin-converting enzyme 2 (ACE2) receptor with the S protein, (B) S protein-mHTCC complex with ACE2 receptor, and (C) S protein-N-carboxymethyl chitosan complex with ACE2 receptor.

4. Discussion

Chitosan derivatives have been reported to influence inhibitors of various human pathogenic viruses, including influenza virus, HIV, Newcastle virus, human norovirus, murine virus, feline calicivirus, hepatitis C virus, Friend murine leukemia helper virus (F-MuLV), herpes simplex virus (HSV), pox virus, and human coronavirus [20,30]. In the present study, we focused on the usage of biodegradable and biocompatible chitosan and its derivatives, which exhibit potent inhibition against spike proteins of coronaviruses. We thoroughly evaluated the effect of molecular weight, configuration, and DS on different monomeric and oligomeric units of chitosan derivatives and their interaction with the spike protein. Milewska *et al.* [17] reported a decline in viral infection rate and multiplication when employing HTCC, a polymeric derivative of chitosan, in the culture medium of human airway epithelium cell lines. HTCC was also identified as a broad-range inhibitor of the other human coronaviruses in its polymeric form, with the DS varying from 57% to 77% [18]. In our study, we evaluated the interaction affinity of the monomeric unit of mHTCC with the spike protein, RBD of the S protein and the ACE2 receptor. The protein-protein interaction study revealed the binding efficiency of mHTCC and the S protein complex with ACE2 receptor, indicating that mHTCC inhibits the binding affinity of the S protein with ACE 2 receptor. These findings are consistent with prior research.

In a recent study by Malik *et al.* [31], trimethyl chitosan (TMC) was identified as a potent chitosan derivative with properties such as high aqueous solubility, stability over a wide range of ionic conditions, and mucoadhesive properties, making it an effective adjuvant molecule. TMC was found to induce strong antibody responses and robust cell-mediated immunity. Alongside TMC, HTCC, a cationic chitosan derivative, has been proven to be effective as an adjuvant when co-administered with the hepatitis E virus (HEV) recombinant polypeptide vaccine through the intramuscular route. Vaccination using HTCC as an adjuvant was associated with an increase in serum HEV-specific IgG antibodies, splenocyte proliferation, and the growth of CD4+ and CD8+ T lymphocytes, and IFN-γ-secreting T lymphocytes in peripheral blood.

These findings suggest that HTCC has a strong immuno-enhancing effect [32].

The current alarming situation necessitates urgent research to proactively address the pathogenesis of SARS-CoV-2 and prevent future outbreaks. The viral entry site is composed of the RBD domain of the S protein (ranges from Arg 319 to Phe591) and the N-terminal peptidase domain of ACE2 receptor (ranges from Ser19 to Asp615). When ligands mHTCC bind with RBD, they interact with Ser438, Asp442, Phe342, Ala344, Asn440, Asn343, Asn437, Thr345, Trp436, Leu441, and Leu441, which fall within the range of amino acids 319–591 of the RBD domain. This region is well-known for its role in binding to host cells. Similarly, mHTCC binds with ACE2 receptor involving Lys562, Tyr196, Gln102, and Ala99, which are part of the virus entry site (**Figure 2**). Moreover, NMNC binds at the RBD site, interacting with Asn343, Arg509, Thr345, Ala344, Leu441, Trp436, Asp442, Ser438, Ser373, Ala372, Ser375, Asn437, Asn440, Ser375, and Phe374 (**Figure 3**). In addition, NMNC binds with ACE2 receptor at amino acids Asn397, Ala396, Arg514, Glu398, Asp206, Gln102, Ser511, Tyr510, Trp203, Tyr199, Gly205, Tyr202, Tyr196, Glu208, Gln98, Leu95, Lys562, and Ala396.

The bindings of mHTCC and NCMC with the RBD involve interactions such as conventional hydrogen bonds, carbon-hydrogen bonds, unfavorable positive-positive interactions, alkyl bonds, pi-sigma bonds, pi-pi T-shaped interactions, van der Waals interactions, and unfavorable donor-donor interactions. Furthermore, the binding of mHTCC and NCMC with ACE2 protein also revealed the presence of conventional hydrogen bonds, carbon-hydrogen bonds, unfavorable positive-positive interactions, alkyl bonds, pi-alkyl bonds, pi-sigma bonds, alternative charges, salt bridges, van der Waals interactions, and unfavorable donor-donor interactions. Similarly, the ligands mHTCC and NCMC, when bound to ACE2 sites, function as the region or medium responsible for the entry of the viral S protein. Our findings also demonstrated strong binding of the ligands mHTCC and NCMC to the RBD site of the S protein. Thus, the chitosan derivatives mHTCC and NCMC demonstrated a dual role, strongly binding to the S protein and impeding viral entry into the host cell ACE2 receptor. These findings align with the research conducted by Milewska *et al.* in 2016 and 2021 [17,18].

In addition to mHTCC, carboxylated chitosan derivatives have demonstrated efficient inhibitory activity against the S protein, yielding binding energies of −7.9, −7.2, and −5.9 kcal/mol for NCMC, N-carboxy ethyl chitosan, and N,O-carboxymethyl chitosan, respectively. These values were ascertained through interactions with the S protein. NCMC exhibited a promising docking score with both the RBD of the S protein and the ACE2 receptor. This promising result paves the way for further investigations, positioning it as a prospective candidate for robust biopolymer development against SARS-CoV-2. Our study's observations reveal the enhanced binding affinity of the carboxylated derivatives of chitosan subunits toward the S protein. Molecular docking studies reveal the efficacy of HTCC, NCMC, N,O-carboxymethyl chitosan, and N-carboxy ethyl chitosan in establishing efficient binding affinity against the S protein of SARS-CoV-2. In this context, the results of this computational investigation indicate that the compounds listed above can be considered for prospective antiviral drugs against SARS-CoV2.

A recent research demonstrated that silymarin-chitosan nanoparticles (Sil-CNPs) can act as a potent antiviral agent for adenovirus 5 (ADV5) and SARS-CoV-2. The researchers evaluated the drug's cytotoxic activity on Vero and Vero E6 cell lines and found it non-cytotoxic while improving its bioavailability and physicochemical qualities. The enhanced antiviral activity of Sil-CNPs may be attributed to their ability to inhibit the viral host ACE2 receptor, thus preventing the virus from attaching to cells [33]. A separate study underscores the reinforcement of bronchoalveolar lavage and local mucosal immunity within the lungs, facilitated by the chitosan-mediated nanovaccine. This innovative approach exhibits the capacity to strengthen the host's defense mechanisms against infection without causing systemic harm [34]. In addition, chitosan/α-Ag_2WO_4 composites emerge as compelling agents with high efficacy in eliminating pathogenic microorganisms, such as *Escherichia coli*, methicillin-susceptible *Staphylococcus aureus*, and the yeast strain *Candida albicans* [35]. These composites also exhibit the ability to inactivate

SARS-CoV-2 on direct contact, achieved through the production of reactive oxygen species. Another study about chitosan aimed to develop mucoadhesive, chemically cross-linked chitosan hydrogels using malic and glutaric acid, without supplementary excipients, solubilizing agents, or catalysts, and examine their interaction with certain natural ACE2 receptor inhibitors [36]. These hydrogels were intended for use as a nasal formulation to reduce the risk of COVID-19 infection. Furthermore, protein-protein interaction studies revealed notable reductions in the binding energy of the S protein with ACE2 in the presence of chitosan and its derivatives. As a result, both compounds are likely to prevent the attachment of the RBD region of S Protein to the ACE2 receptor [37,38]. It is important to note that, however, these findings necessitate further experimental validation. The carboxylic group emerges as an important structural feature that contributes to the heightened affinity of chitosan for this target. These groups also exhibit the capacity to enhance water solubility and flocculating capacity, which may enhance the *in vivo* efficacy [5].

5. Conclusion

The novel COVID-19, responsible for the recent pandemic, stands as a global threat with no established curative drug. Instead, the predominant approach involves managing the illness through supportive therapies employing repurposed drugs. Notably, NCMC, mHTCC, and various biocompatible chitosan derivatives have exhibited significant antiviral activity *in-silico*. These compounds hold potential as inhibitors of SARS-CoV-2 infections. Within this context, the results of this computational investigation propose that NCMC and mHTCC warrant further investigation as potential contenders for future antiviral medications targeting SARS-CoV2. Nonetheless, it is important to underscore that additional experimental validation is imperative to corroborate these findings.

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Conflict of interest

The authors declare no conflicts of interest.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

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Availability of data

Not applicable.

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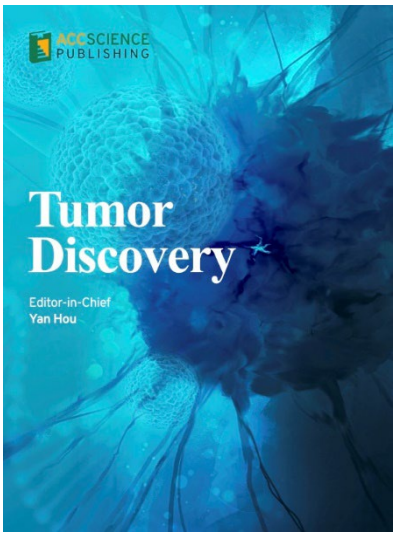

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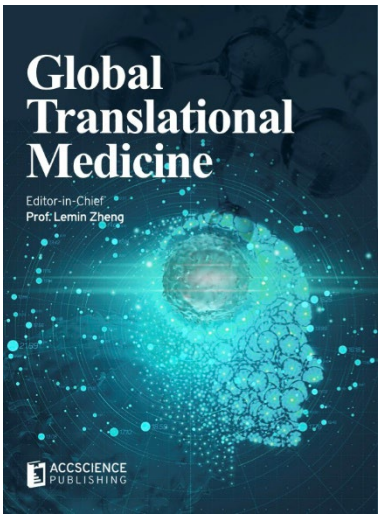

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