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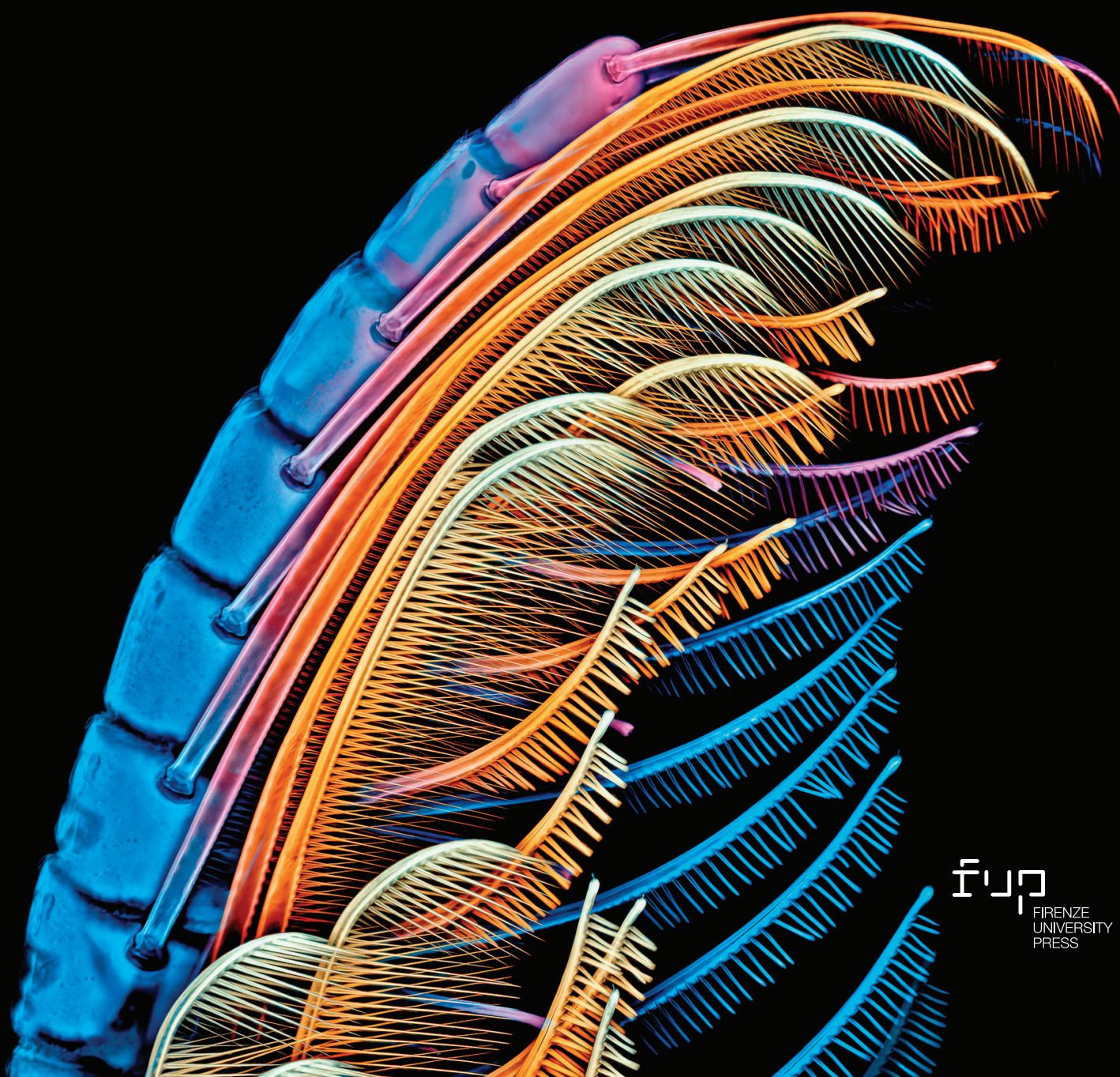
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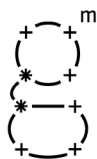
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Editorial

The Origin of Sugars

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Life as we know it on Earth is anchored by four fundamental classes of biological macromolecules. These pivotal, carbon-based molecular pillars include proteins (based on amino acid), DNA and RNA (whose “letters” are represented by nucleobases), lipids and carbohydrates. The last named form the backbone of DNA and RNA and play a fundamental role in metabolic processes.

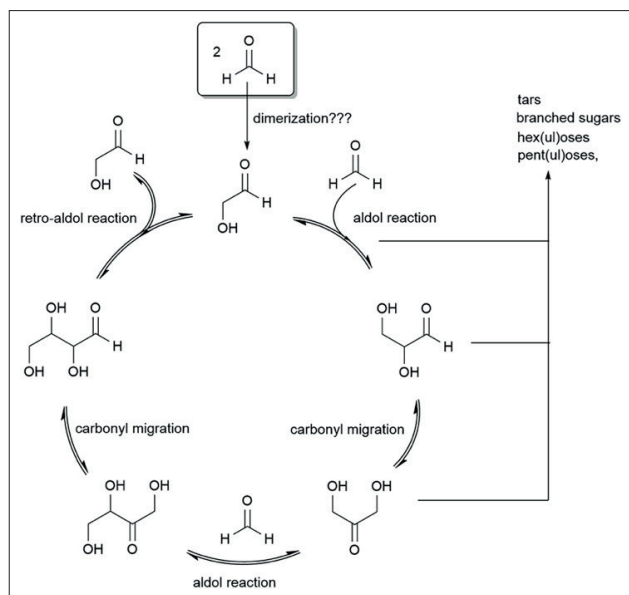


Figure 1. The initial formation of glycolaldehyde from glyoxylate is followed by a series of repetitive (1) aldol condensations with glyoxylate, (2) carbonyl migrations, and (3) decarboxylations, without the disadvantages of formaldehyde-based reactions.

One of the main questions about the origins of life is how these molecules formed. In laboratory experiments, simulating prebiotic conditions yield only low

concentrations of carbohydrates, certainly insufficient to support the pivotal role that they actually play. Many scientists believe that the first sugars originated from reactions involving formaldehyde—specifically the “formose” reaction (crasis of “formaldehyde” and “hexose”). However, this molecule triggers uncontrolled side reactions, and its high reactivity, under the conditions hypothesized for the early Earth, presents further challenges. A second theory was formulated in 2007: a scenario based on the “glyoxylate reaction,” in which glyoxylate first reacts with itself to form glycolaldehyde. Researchers suggest that glyoxylate, glycolaldehyde, their byproducts, and other simple compounds could have continued to react with one another, finally producing simple sugars and other products [1].

Alongside the hypothesis that carbohydrates formed on Earth, there is another hypothesis suggesting an exogenous origin. This hypothesis is supported by the detection of sugars essential for life, such as ribose and glucose, in meteorites and, more recently, on the asteroid Bennu [2] and on Arrokoth, a Kuiper belt icy body originated during the formation of the Solar System 4.6 billion years ago [3]. Did these sugars form on these minor bodies, or, more likely, they were present in the cloud from which the solar system originated? To Answer this question requires detecting sugars and/or their precursors in the interstellar medium (ISM). The ISM is a remarkable chemical factory, where over 340 molecules have been detected, including large aromatic species. Several of these molecules—such as urea, hydroxylamine, and ethanolamine—are directly linked to the chemistry of the origin of life, as they are considered precursors to ribonucleosides and lipids. However, no sugars had been observed to date. A major obstacle in searching for interstellar sugars has been the lack of data

regarding energy transitions between quantized rotational states in the gas phase. Such data are essential for astronomical identification via radio telescopes, but they are extremely difficult to obtain, as sugars are thermally fragile and highly hygroscopic—properties that hinder their handling and conventional thermal vaporization.

This limitation has recently been overcome thanks to ultrafast laser vaporization, which enabled the gas-phase rotational characterization of ribose and 2-deoxyribose, as well as erythrulose. It is precisely the last one that was recently detected through ultrasensitive, broadband spectral surveys of the molecular cloud G+0.693–0.027 in the Galactic Center of the Milky Way (Figure 2)—located approximately 26,000 light-years away—using the Yebes 40 m and IRAM 30 m radio telescopes (Figure 3) [3].

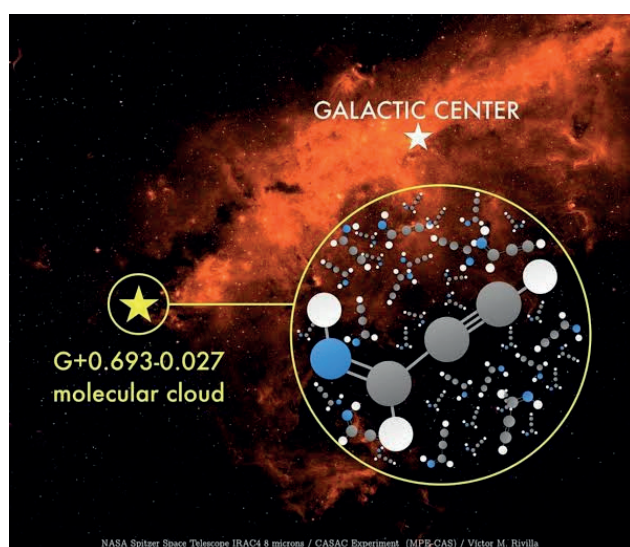


Figure 2. The G+0.693–0.027 molecular cloud is located within the Sgr B2 star-forming complex; it spans 150 light-years and has a mass 3 million times that of the Sun, situated at the center of our Galaxy. Copyright NASA.

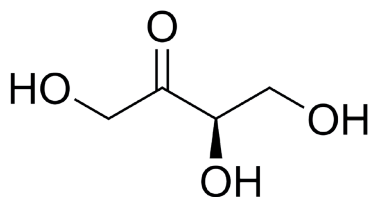


Figure 3. Structural formula of erythrulose, IUPAC name (R)-1,3,4-trihydroxybutan-2-one

Erythrulose, with 14 atoms in its structure, represents the largest non-cyclic molecular species identified to date in the ISM and is the first detected molecule

containing four oxygen atoms. It is also the first sugar and the second chiral molecule reported in the ISM, following propylene oxide. Its presence in the interstellar medium not only provides direct evidence that complex, chiral species can form under interstellar conditions, but also advances our understanding of the scale of interstellar chemical complexity, suggesting that other prebiotic (and potentially chiral) molecules could form and survive even in these extreme conditions. This sugar can serve as a basis for the formation of ribonucleotides—the building blocks of RNA. Ketoses like erythrulose can readily isomerize into their corresponding aldoses (namely, threose and erythrose) in aqueous environments, a transformation crucial for the formation of ribose. What is the pathway in the ISM for synthesizing this carbohydrate? Recent laboratory experiments of irradiated methanol ices find out that sugars with up to six carbon atoms can be obtained from smaller sugar and sugar-derivative fragments (e.g. C3 sugar precursors) [3]. In this molecular cloud the C3 sugars glyceraldehyde and dihydroxyacetone are at least 8–17 times less abundant than erythrulose, suggesting that they are not dominant precursors. More abundant building blocks of this C4 sugar are present as glycolaldehyde and ethylene glycol in G+0.693. Therefore, these substances can be the precursors of Erythrulose on the surface of interstellar dust grains. Consequently, once formed, erythrulose and other sugars could be incorporated into the planetesimals that give rise to bodies in the outer Solar System, such as Kuiper Belt objects.

The discovery of chiral erythrulose supports also the idea that small enantiomeric excesses measured in meteorites (on the order of a few percent) may originate in extraterrestrial environments, which could have contributed to the emergence of biological homochirality on the early Earth, through subsequent chemical amplification processes. Thus, erythrulose might have arrived on Earth during the Late Heavy Bombardment between 4.1 and 3.9 billion years ago. Although the extent and intensity of the Late Heavy Bombardment have recently become questionable, simulations of Earth's impact history show that biopoiesis—the process describing the development of living matter from non-living organic matter—could have occurred over a much broader time-frame, between 4.45 and 3.9 billion years ago. Since glycolaldehyde, small sugars, and sugar derivatives have been shown to survive meteorite impacts, the presence of erythrulose in interstellar space reinforces the hypothesis of an exogenous origin for the sugars relevant to the synthesis of early nucleic acids. The presence of erythrulose on the surface of the early Earth and its rapid isomerization into threose (Figure 4) support its involvement in the formation of threose nucleic acid

(TNA), a structurally simpler RNA analogue [5]. Threose nucleic acid has been synthesized in the laboratory and, in the context of the origin of life, has been proposed as one of the polymers that may have played a role in a pre-RNA world.

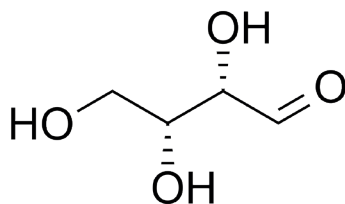


Figure 4. Structural Formula of Threose

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- 3) Zhang C. et al. (2024) Ionizing radiation exposure on Arrokoth shapes a sugar world *PNAS*, 121, e2320215121 <https://doi.org/10.1073/pnas.2320215121>
- 4) Jiménez-Serra I et al (2026) Detection of a four-carbon sugar in interstellar space *Nat. Astron.* <https://doi.org/10.1038/s41550-026-02905-7>.
- 5) Threose nucleic acid (TNA)—or (L)-α-threofuranosyl nucleic acid—is a nucleic acid similar to DNA and RNA but differs from them in the composition of its backbone, which consists of repeating threose units linked by phosphodiester bonds, rather than ribose or deoxyribose.



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Research Article

Peter Griess (1829-1888) – Breweries, Diazonium Salts, Dyes

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Abstract. Peter Griess was probably the chemist who exercised both sides of his profession with equal effectiveness: the rigorous researcher, and the industrial chemist, carrying out his duties with absolute seriousness and efficiency. After a rather hectic period in life, Griess began a scientific activity of extreme importance in Organic Chemistry: the discovery and study of diazonium salts, and work with dyes, aromatic compounds, and other nitrogenous compounds, an activity carried out mostly outside the academic context. His practical activity in the brewing industry is less publicized, due to professional secrecy, but no less interesting and varied. This dual activity is presented in the historical context in which it occurred, whenever possible based on primary sources.

Keywords: Peter Griess, Diazonium salts, Dyes, Aromatic compounds, Breweries, Royal College of Chemistry

“And since then Griess’s reactions have become so commonplace that enumerating them might seem an unnecessary task.” (Emil Fischer)¹

INTRODUCTION.

Among the less remembered – but no less important – organic chemists of the second half of the 19th century, we recall, among others, Peter Griess, a combination of industrial chemist and researcher, known for the discovery of diazonium salts, of such importance in Organic Chemistry, both in the synthesis of dyes and substituted aromatic compounds. He successfully fulfilled his dual role as an industrial chemist in the brewing industry and as a researcher in Organic Chemistry. Like other German chemists, such as Ludwig Mond, Carl Schorlemmer, and Hugo Müller, he settled permanently in Great Britain (1858), where he started a family (1869) in Burton-on-Trent. He died prematurely in Bournemouth in 1888. In 1891, his colleagues and friends August Bopp (1833-1904), August Wilhelm von Hofmann (1818-1892), and Emil Fischer (1852-1919) published a lengthy article in *Berichte*, “Zur Erinnerung an Peter Griess” (Remembering Peter Griess).² F. A. Mason

published, in 1930, “Johann Peter Griess 1829-1888”³; There are several concise articles on the life and work of Griess, all drawing from the rich source that is the rather personal article by Bopp, Hofmann and Fischer, and of which we highlight those by V. Heines in the *Journal of Chemical Education* (1958)⁴, Robert Wizinger-Aust (1896-1973) in *Angewandte Chemie* (1958)⁵, and W. H. Cliffe in *Coloration Technology* (1959)⁶. Edwin Yates and Andrew Yates wrote about Griess and the brewing industry (2016)⁷. This article aims to include other sources and new connections between Griess and Chemistry.

ORIGIN, YOUTH, STUDIES.

Johann Peter Griess was born on September 6, 1829, in Kirchholzbach, a small village of 200 inhabitants near Kassel, the son of Johann Heinrich Griess (1799-1870) and Catherine Elisabeth Griess (1803-1860). His father was a small farmer and blacksmith in the village, and owned a small stretch of forest from which he extracted timber, including the wood to build his own house. After primary school in the village, his father, who wanted his son to one day take over his small property, sent him in 1844/1845 to a private agricultural school in Beberbeck (now part of the city of Hofgeismar), but Peter had no interest whatsoever in agricultural activities. He then attended the *Höhere Gewerbeschule* (Higher Trades School) in Kassel, a technical education center founded in 1831 by Friedrich Wöhler (1800-1882), where he had his first contact with science (he had learned the rudiments of botany from the teacher in his native village), with professors Heinrich Buff (1805-1878), a physicist, and Amandus Philippi (1808-1904), a zoologist, who after the liberal revolution of 1848 emigrated to Chile, where he became a professor of Zoology at the University of Santiago.

He later attended the universities of Jena (1850/1851), Marburg (1851), where he attended the Philosophy lectures of Eduard Zeller (1814-1908), a follower of Kant, Munich (1854), where he was allegedly a student of Liebig, without official enrollment, and again Marburg (1854), as a student of Hermann Kolbe (1818-1884). Initially, he did not take academic life very seriously, incurred debts paid by his father, was imprisoned in the university jail (*Karzer*, common at the time) for disturbing the public order, and ended up suspended from Marburg, but he used the time to improve his deficient humanistic education.



(Fig.1) Peter Griess (1829-1888). Photograph by an unknown author, c.1885. Illustration from R. Anderson, “The Brewing Connection in the Oxford Dictionary of Biography” (public domain).



(Fig.2) Hermann Kolbe (1818-1884), Griess's professor in Marburg. Photograph by August Brasch, Leipzig, 1879 (Wellcome Foundation, London).

In 1856, temporarily away from the university and without resources, he found employment in industry, through A. W. Hofmann, at *Teerfarbenwerk Oehler* in Offenbach, which manufactured dyes from tar and residues from the Frankfurt gasometer. Karl Gottlieb Oehler (1797-1874) had acquired the factory in 1850 from its founder, the chemist Ernst Sell (1808-1854), a student of Liebig and a pioneer in the industrialization of tar in Germany, with the factory he founded in 1842 in Offenbach⁸. (Sell sent the first samples from his factory to Liebig, in whose laboratory A. W. Hofmann redis-

covered aniline (*Kyanol*) and Friedlieb F. Runge's [1794-1867] quinoline in 1842). The factory was destroyed by a fire in 1857, and Griess returned to Marburg and his mentor Kolbe, somewhat against the latter's wishes, but now demonstrating a great interest in chemistry and an unusual desire to research. In Marburg, Kolbe suggested to Griess to carry out the reaction of nitrous acid with aromatic amines, since experiments by Wurtz (1851)⁹ and Hofmann (1855)¹⁰ had already shown that aliphatic amines decompose into alcohol and nitrogen when reacting with nitrous acid. The reaction of nitrous acid with aromatic amines led to the discovery of diazonium salts (1858) by Griess, who published a "provisional notice" about them in the *Annalen* (1858)¹¹. This fell into the hands of Hofmann at the *Royal College of Chemistry* in London, and Hofmann, the director of the *College*, knowing of Griess's unemployment and financial precariousness, and heeding a request from Kolbe, invited him to be his assistant. Hofmann had realized (due to his experiments with coal tar derivatives Hofmann had a wider view of the future of synthetic dyes), before Griess himself, the scope of the discovery, translated Griess's article, added new discoveries by Griess, and published it¹². The various biographers of Griess are more or less benevolent regarding his time as a student in Marburg, considering that Griess became a serious and tireless worker, always willing to help those in need, and an exemplary family man. In Wizinger-Aust's opinion, the life and work of Peter Griess was still not properly valued by historiography, even to his days. Emil Fischer's assertion from the epigraph has lost some of its relevance today.



(Fig.3) August Wilhelm Hofmann (1818-1892), from an article by Edward John Hallock, published in *Popular Science Monthly*, volume 24, edition from April 24, 1884.

ENGLAND, THE ROYAL COLLEGE OF CHEMISTRY AND THE BREWERIES

For someone in Griess's difficult situation, Hofmann's invitation was irresistible. Griess accepted it, and by December 1858 he was already working at the *Royal College*. The *Royal College of Chemistry* had been founded in 1845 by a group of benefactors, with the aim of training the chemists needed for the growing Industrial Revolution; but the direct profits from research carried out there would also be welcome.... Among the members of the first board of directors were: Alexander Baring, Lord Ashburton (1774-1848), financier and politician, Sir James Clark (1782-1870), personal physician to Queen Victoria, Sir William Tite (1798-1873), architect, Warren De La Rue (1815-1889), chemist and industrialist. The cornerstone of the *College's* headquarters on Oxford Street, designed by James Lockyer (1796-1875), was laid on June 16, 1846, in the presence of Prince Albert (1819-1861), civil and religious authorities, and scientists (Graham, Brande, Hofmann, Wheatstone). The first director was August Wilhelm Hofmann, whose stay in England (he returned to Germany in 1865 to take up the chair of Chemistry at the University of Berlin) was supported by Prince Albert and the King of Prussia, Frederick William IV (1795-1861)¹³. Among the alumni of the *College* were Sir Frederick A. Abel (1837-1898), John Alexander R. Newlands (1837-1898), Sir William Perkin (1838-1907), Charles Mansfield (1819-1855), John Stenhouse (1809-1880), and Edward Divers (1837-1912), who was a professor in Japan from 1873 to 1899.



(Fig.4) The old laboratory building of the Royal College of Chemistry, by James Lockyer (1796-1875), begun in 1846, with the presence of Prince Albert. Lithograph by Day & Haghe (Wellcome Foundation, London).

Peter Griess remained at the *Royal College of Chemistry* from 1858 to 1861, continuing his research on diazonium salts, about which he published a new article in 1860¹⁴. But Griess's first scientific work produced there deals with the introduction of iodine into certain aromatic compounds¹⁵, also involving diazonium salts and in a way anticipating the future (1884) Sandmeyer reaction (Traugott Sandmeyer, 1854-1922), of the Zurich Polytechnic. Other work carried out at the *Royal College* addresses the nitrogenous derivatives of aniline and its homologues (1861)¹⁶, the action of nitrous acid on diamines (1862)¹⁷, and the production of non-nitrogenous acids from amines¹⁸. With Carl Adolph Leibius (1833-1893), Hofmann's assistant from 1857 to 1859 (when Leibius emigrated to Australia), he studied the action of HCN on methylamine, with the formation of methylurea¹⁹. This research was important for understanding cyanamides as fertilizers. In London, Griess met Heinrich Caro (1834-1910), one of the most important chemists involved in the synthesis of dyes, then working at *Roberts, Dale & Co.* in Manchester, and after his return to Germany (1866) technical director of *BASF* (where Griess would later patent his dyes). The friendship between Griess and Caro also resulted in a scientific partnership, which, however, was less productive than might have been expected.

GRIESS AND ALLSOPP & SONS

In 1861, at Hofmann's house, Griess met the German chemist Wilhelm Heinrich Böttinger (1820-1872), a student of Liebig, who from 1845 to 1865 (when he returned to Germany) was chief chemist of the large *Allsopp & Sons* brewery in Burton-on-Trent, a major brewing center near Nottingham. This meeting resulted in Griess being hired by Allsopp in 1862; Griess was a chemist and later chief chemist of the company until his death in 1888. Böttinger, a native of Heilbronn, was, on Liebig's recommendation, an assistant in Glasgow, then a chemist at *Allsopp*; returning to Germany, he turned his interests to research, in the laboratories of Lambert von Babo (1818-1899) in Freiburg, Emil Erlenmeyer (1825-1909) in Munich, and in Würzburg. He later founded a small brewery in Würzburg, where he died²⁰.

The friendship with Böttinger was beneficial to Griess, both when he moved to Burton (180 km from London), in the social contacts at his new workplace, and in the translation of his articles into English. After Böttinger left, Griess met the family of the physician Francis Albert Mason, whose daughter Louise Anna (1839-1886) he married in 1869. The couple had two sons and two daughters.

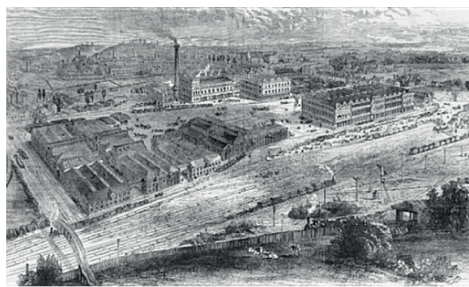
Upon joining *Allsopp*, Griess found other chemists working in the city: Horace Tabberer Brown (1848-1925), Adrian John Brown (1852-1919), both natives of Burton, and the Irishman Cornelius O'Sullivan (1841-1907), who worked at *Bass & Co.* (a company founded in 1777 by William Bass [1717-1787]). O'Sullivan had been a student of Hofmann at the *Royal College of Chemistry* and an assistant to Hofmann in Berlin). In 1876 Griess, the three other Burton chemists, two physicians and two brewers founded the "Bacterium Club", an informal gathering for the discussion of novelties in chemistry and biology.

The *Allsopp & Sons* brewery in Burton-upon-Trent traces its origins back to 1743 with the tavern owner and brewer Benjamin Wilson. In 1800, his nephew Samuel Allsopp (1780-1838) joined as a partner. In 1807, due to difficulties arising from Napoleon's Continental Blockade, Wilson sold the company to Allsopp. In the 1860s, it became the second largest brewery in Great Britain, operating as *Samuel Allsopp & Sons Limited* since 1887. In the 20th century, it faced significant financial difficulties and only returned to operating under its former name in 2020. When Griess joined the company, it was run by Sir Henry Allsopp (1811-1887), who was also active in politics.

The scientific study of the art of brewing began in the 19th century, during the time of Griess, not only from a chemical point of view, but also from a biological one (organisms and processes responsible for fermentation, a chemical reaction that was only then beginning to be clarified²¹, with the proposals of Pasteur, Liebig, Vicente Marciano [1848-1891] and finally Eduard Buchner [1850-1917] in 1897). The extensive research of the Carlsberg laboratory in Copenhagen also falls within this period. Quality control, which went hand in hand with potential for adulteration, also fell to the brewery chemists. For example, in 1852 Anselme Payen (1795-1871), professor at the *Conservatoire des Arts et Métiers* in Paris, recklessly accused English breweries of adding strychnine to the beverage to increase its bitterness. The "strychnine scandal" shook sales of Allsopp's famous *Pale Ale*, and it required the intervention not only of Böttinger, but also of Graham, Hofmann, and Liebig, who concluded in letters to the company that the accusation was false, but doubts about the case remain even today²². Graham and Hofmann published an extensive report in 1852 on their analyses of beers from many Burton breweries, concluding that "none of these varieties of beer, when tested with the greatest scrupulousness, gave the slightest evidence of the presence of strychnine"²³. According to the report, Payen apologized, claiming that similar suspicions had been raised in France, in a book by the pharmacist Jean-Baptiste Chevallier (1793-1870)²⁴. A year earlier, an investigation into food adulteration

and falsification had taken place. The investigation also included, among others, the company *Bass, Ratcliff and Gretton*, founded in 1777, also in Burton. The problem of adulteration and contamination not only of beverages but also of food was quite serious at the time, and the strychnine case caught the attention of Charles Dickens (1812-1870), who commented on it in “Household Works”, also mentioning the adulteration of beers with picrotoxin²⁵, a highly toxic substance extracted in 1812 by Pierre Boullay (1777-1869) from *Anamirta cocculus*, a plant from Southeast Asia, added to accentuate the bitter taste and increase the intoxicating effect of beer.

Griess's activity at Allsopp is quite strange. He was not very involved with the chemistry related to beer production, but he carried out research of personal interest in the company's laboratory, such as the reactions of diazonium salts and the obtaining of dyes, which, when useful in the industry, were patented, not in Great Britain, but in Germany by German companies, such as *BASF*, and for Griess's own benefit. It is difficult to believe that Allsopp gave him so many privileges; explanations could be found in Griess's great prestige in the chemical field, as well as in the patent legislation in both countries (in Germany there was only unified legislation after 1871). When the demand for beer increased in the 1870s (*India Pale Ale*, a more bitter beer, richer in hops and with a high alcohol content, was exported worldwide), the company demanded greater involvement from Griess in the manufacturing aspects of the product, which did not please the chemist at all. However, Griess remained with the company until the end of his life. Few records exist about Griess's activity at Allsopp, but it must have been very fruitful, considering the quantitative and qualitative development of the company's production. The freedom he was granted in the laboratories also speaks positively about his activity in the brewing industry. The investigations and improvements he introduced to the brewing industry remained largely secret.



Allsopp's New Brewery from *The Illustrated London News*, 1 November 1862.



(Fig.5 - Left). Samuel Allsopp & Sons brewery, in Burton-on-Trent. From *The Illustrated London News*, November 1, 1862. (Right) Samuel Charles Allsopp, 2^o Baron Hindlip (1842-1897), caricature by Sir Leslie Ward (1851-1922), published in *Vanity Fair*, August 1885.

GRIESS, DIAZONIUM SALTS AND AZO COMPOUNDS.

Diazonium salts constitute one of the most important groups of organic compounds, as they are the basis of two important series of reactions: by coupling, the obtaining of a large number of azo compounds, among which are many dyes, a field of study of Griess himself; and a means of easily introducing substituents into aromatic rings, a method discovered in Zurich by Traugott Sandmeyer (1854-1922) in 1884 and known as the Sandmeyer Reaction (there are many variants of this reaction, such as the Balz-Schiemann Reaction [1927], for the introduction of fluorine)²⁶. Diazonium salts were discovered by Griess in 1858, while still at the University of Marburg, as already mentioned. Some authors cite as a distant inspiration a reaction discovered by Raffaele Piria (1814-1865), who obtained alcohols by treating primary amines with nitrous acid (1846)²⁷. Griess published his discovery in a “provisory note” in Liebig's *Annalen*. Diazonium salts are obtained by treating aromatic amines with nitrous acid (obtained in situ from nitrites and HCl), which precipitates yellowish crystals of diazonium salts that are purified by recrystallization. The salts are unstable at room temperature, are kept chilled, and used in situ, without being isolated from the mixture. The reaction became known as “diazotization.” But this simple diazotization method was only developed in 1873 by Victor Meyer (1848-1897). The path followed by Griess to arrive at diazonium salts was quite tortuous, and since the formula for benzene proposed by Kekulé, dating from 1865, was not yet known, it was also difficult to propose a structure for the salts. Only after the diazotization of aminonitrobenzoic acid and aminodinitrobenzoic acid did Griess perform the diazotization of aniline. Griess does not state in his publication why he did not begin his experiments with aniline. His articles are concise, without details regarding the experimental procedure. He initially obtained diazoaminobenzene, which he treated with bromine and nitrous acid in the presence of nitric acid:



According to Wizinger-Aust, these are the first true diazonium salts.

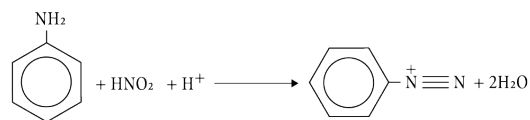


Fig. 6 Synthesis of diazonium salts

The structure of these compounds remained unknown for a long time, and several chemists proposed formulas for them, such as those in Figure 7 below, suggested by Griess himself, by Kekulé, and by Blomstrand. Elemental analyses of the compounds showed only the presence of two nitrogen atoms, but it was not possible to determine their position in the structure.

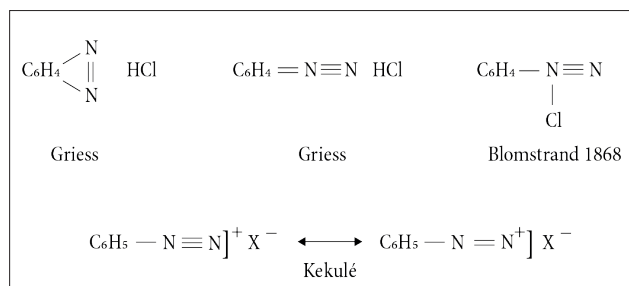


Fig. 7 Representations of diazonium salts

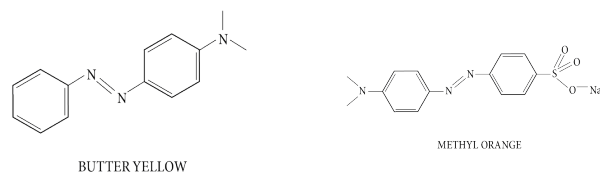
After diazotization of aminonitrobenzoic acids and aniline, Peter Griess wanted to determine the limits of his new reaction and studied the possible diazotization of naphthalene, naphthols (which both do not react), sulfanilic acid, benzidine, toluidine, etc., starting in 1858, communicating the results in 15 publications, and arriving at a general understanding of the “coupling” in 1864, concluding in 1875 with a summary in the *Berichte*.²⁸ In his papers, Griess defined “coupling” as the direct linkage of diazonium compounds with amines or phenols.

Griess dedicated his entire life to diazonium salts, working alone, and his study of these compounds was so thorough that for a long time, during Griess’s activity, there was little to add. Among future additions was the formation of azo compounds with an aromatic and an aliphatic chain²⁹, discovered in 1875 by Victor Meyer, who also determined the structure of these compounds³⁰. In 1882, Victor Meyer and Emil Constan (1858-1917), in Zurich (Constan’s doctoral thesis), believed they had obtained purely aliphatic azo compounds³¹. However, Heinrich Wieland (1877-1957) showed that it was nitrohydrazine. In 1910, Johannes Thiele (1865-1918) succeeded in obtaining purely aliphatic azo compounds³².

With the discovery of diazonium salts, the study of these compounds led Griess to azo dyes, an important group of dyes containing one or more N=N= groups. Azo dyes are obtained by the coupling reaction of diazonium salts with aromatic amines, and the first azo dye was obtained by Griess himself in 1861: aniline yellow or butter yellow, by reacting a diazonium salt with dimethylaniline. (some historians mention a simultaneous discovery by the French chemist Charles Mène).

Butter yellow began to be produced commercially from 1864 by the company *Simpson, Maule & Nicholson*, in Locksfield, near London, and was used in the coloring of foods and pharmaceuticals, for example, to impart a yellowish color to margarine, to make it more similar to butter. This use was prohibited after the discovery of the carcinogenic activity of this dye in 1937 by Rioju Kinoshita (1893-1977) at Osaka University. The company *Simpson, Maule & Nicholson* was founded in 1853 by George Simpson (1819-1894), a merchant of scientific equipment, George Maule (1827-1890), a chemist, and Edward Chambers Nicholson (1827-1890), a student of Hofmann at the *Royal College of Chemistry*.

Another important azo dye in the history of Chemistry, developed in 1875 by Griess (independently and almost simultaneously by Nikolaus Otto Witt [1876] and Zacharie Roussin [1878]), is methyl orange (Goldorange, Orange III, Tropeolin). Georg Lunge (1839-1923), a professor in Zurich, introduced it into Analytical Chemistry as an indicator for the pH range of 3.1 to 4.4. Griess also developed other azo dyes, such as Chrysoidin (1876, also by Heinrich Caro), chrysoin (1875), Orange I and Orange II (1876).



Both at the *Royal College of Chemistry*, as Hofmann’s assistant, and in the Allsopp laboratories, Griess prepared a large number of azo dyes, which were sent to Dresden to be analyzed by his friend Rudolf Schmitt (1830-1898), professor of Organic Chemistry at the Dresden Polytechnic. Walther Hempel (1851-1916), Schmitt’s assistant and later professor of Technological Chemistry at the Dresden Polytechnic, recounts these discoveries: “The new compounds prepared by Griess were regularly sent, in small packages, from Burton-on-Trent to Dresden for analysis. At the same time, these shipments were accompanied by welcome barrels of the finest Pale Ale beer.”³³ As was customary at the time, the analyses were likely performed by Schmitt’s assistants, like Hempel, though Hempel also published analyses of his own³⁴. It is probable that Schmitt performed some analyses himself, given the close friendship between Griess and Schmitt. Since 1880, many dye industries regularly sent samples for analysis in Dresden, by Professor Rudolf Schmitt, and later by Professor Walter König (1878-1964). The Technical University of Dresden still holds a

large collection of dyes (samples of 12,000 synthetic dyes and 1,500 natural dyes), founded in 1926 by Professor Richard Möhlau (1857-1940).

Dyes developed by Griess

1875 Methyl Orange or Aniline Yellow

1875 Chrysoin

1876 Chrysoidin (also by H. Caro, 1875)

1876 Orange I

1876 Orange II

With these discoveries and syntheses, Peter Griess was an exponential figure in the development of synthetic dyes, although he himself had little advantage from these discoveries. Heinrich Caro (1834-1910), an undisputed leader of the nascent dye industry, said of him: "For the industry, he was a teacher and leader, but others reaped where he sowed; Griess was not a successful inventor."

Regarding a supposed isomer of alizarin.

Alizarin is a natural dye used since antiquity, obtained from the madder root (*Rubia tinctoria*), from which it was isolated by Colin and Robiquet in 1825. In 1868, Carl Graebe (1841-1927) and Carl Liebermann (1842-1914) succeeded in synthesizing alizarin from anthraquinone³⁵. For this reason, they determined its partial structure (dihydroxyanthraquinone). The definitive structure (1,2-dihydroxyanthraquinone) was clarified in 1874 by Adolf von Baeyer (1835-1917) and Heinrich Caro (1834-1910). This brief account shows that ignorance of a compound's structure leads to the formulation of hypothetical reactions, seemingly logical, but mistaken; we could say, in logical terms, that the premise is wrong. Thus, in 1850, Julius Wolff and Adolph Strecker (1822-1871) believed it would be possible to synthesize alizarin from naphthalene³⁶, based on the fact that alizarin and chloro-oxy-naphthalenecarboxylic acid, when heated with nitric acid, yield the same products: phthalic acid and oxalic acid. In practice, alizarin was not obtained. In 1865, Griess and Carl Alexander von Martius (1838-1920) revisited the subject³⁷, and starting from dinitronaphtyl alcohol and going through a reduction step (converting the groups $-\text{NO}_2$ in $-\text{NH}_2$) and successive decompositions, they obtained a compound with the formula $\text{C}_{10}\text{H}_6\text{O}_3$. Considered an isomer of alizarin (the molecular formula would be the same), this new compound does not exhibit the coloring properties of alizarin. The history of Chemistry shows that it was possible to synthetically reproduce a natural compound without knowing its structural formula (Graebe and

Liebermann synthesized alizarin knowing its structure partially), but these syntheses were exceptions. Griess's previous empirical work on azo compounds and picric acid simultaneously and gradually involved the structure of the participating compounds, and a careful interpretation of the empirical data allowed him to arrive at the structures in question. This is not the case with alizarin, which is discussed here.

OTHER EXPERIMENTAL WORKS BY GRIESS.

Peter Griess' experimental work focuses on the obtaining and investigation of azo compounds, with a high point in dyes, but there is also a great contribution to the chemical study of aromatic compounds. The research related to brewing is unprecedented because it was of industrial interest. There is only one publication by Griess (with G. Harrow) in this area, in the *Berichte* of the German Chemical Society, on the occurrence of choline in hops and beer, a publication of a more general scientific nature³⁸. His articles are scattered in many periodicals, mainly in the *Berichte* of the German Chemical Society and in Liebig's *Annalen*, but also in the *London Philosophical Transactions*, the *Journal für praktische Chemie*, and the *Zeitschrift für Chemie und Pharmacie* (edited by Emil Erlenmeyer). Let's look in detail at some of these investigations.

The Griess test and the Griess reagent.

The Griess test is used for the detection of nitrites in biological fluids and drinking water, and also for the detection of nitrates, properly reduced to nitrites, in explosives, a method used in forensics (detection of nitroglycerin). The Griess reagent consists of one part naphthylethylenediamine chloride in a 0.1% aqueous solution and 1% sulfanilamide in 25-50% acetic acid. The nitrite ion reacts with sulfanilamide, giving rise to a diazonium salt, which reacts with naphthylethylenediamine chloride in a coupling reaction, giving rise to a pink or red solution, the concentration of which is determined by spectrometry. The Griess test is based on a coupling reaction and was described in detail by Peter Griess in 1879. Instead of sulfanilamide, he used sulfanilic acid.

There are commercially available variants of the Griess reagent. In 1889, Casimir Wurster (1854 Weidenthal/Rhineland – 1913 Dresden), a student and assistant of Victor Meyer in Zurich and of Rudolph Fittig in Strasbourg (1877/1878), developed a very practical method for performing the Griess test, using "indicator

papers”: a yellow reagent paper impregnated with alpha-sulfanilamide and a red reagent paper impregnated with sulfanilic acid. Using both together detects nitrite. Wurster extended the method to detect the nitrogen oxide formed in the determination of HNO_3 with iron sulfate, FeSO_4 in the presence of ammonium acetate, which was once important for water quality control³⁹. Wurster also developed several other analytical reagents, such as redox indicators (Wurster blue) and reagents for use in industrial analyses.

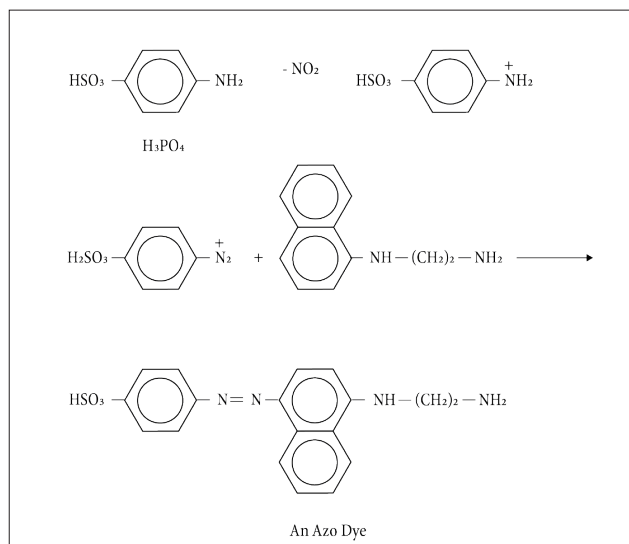


Fig. 8 Reaction of the Griess test

The structure of disubstituted benzene derivatives.

Wilhelm Körner or Guglielmo Koerner (1839-1925), professor at the Higher School of Agronomy of the University of Milan, is generally credited with elucidating the structures of the three disubstituted derivatives of benzene, in this case the structure of the three dibromobenzenes (1874). To do this, he performed the nitration of these compounds: the nitration of 1,2-dibromobenzene yields two nitrodibromobenzenes, the nitration of 1,3-dibromobenzene yields three derivatives, and the nitration of 1,4-dibromobenzene yields only one nitrated derivative. The careful separation of the isomers obtained thus allows the determination of the structure of ortho-, meta-, and para-dibromobenzene. The prefixes ortho-, meta-, and para- were proposed by Körner. Partington, in volume IV of his “History of Chemistry”, attributes this proposed nomenclature to both Koerner and Carl Graebe (1841-1927)⁴⁰.

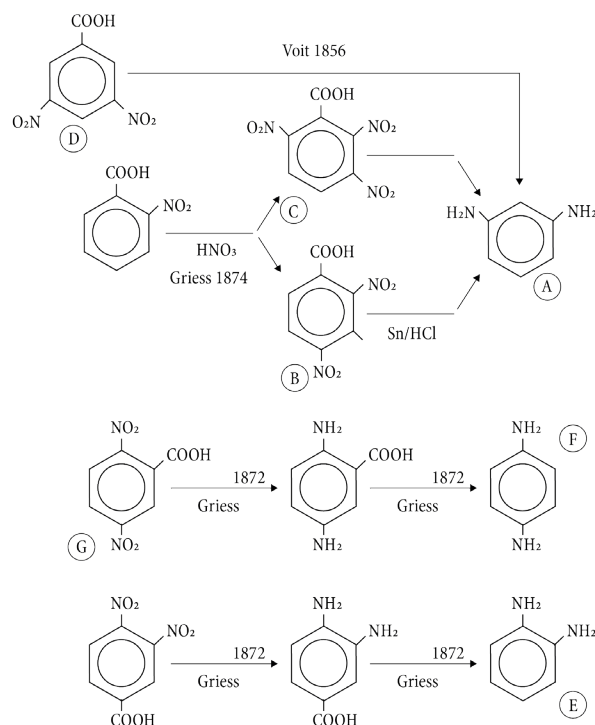


Fig. 9 Reactions involving dinitrobenzoic acids

In fact, Peter Griess had already clarified the structure of the three disubstituted benzene derivatives in 1872, but through reverse reasoning: he decarboxylated and reduced the six dinitrobenzoic acids, obtaining the three phenylenediamines (melting points 63°C, 99°C [current value 103°C] and 140°C). The sequence of experiments performed by Griess and the reasoning that followed to arrive at the structures of the three disubstituted benzene derivatives are more complex and involve previous experiments by other researchers. In 1849, Auguste Cahours (1813-1893) obtained a dinitrobenzoic acid⁴¹ (we know today that it was 3,5-dinitrobenzoic acid), which Carl von Voit (1831-1908), a student of Liebig and Pettenkofer in Munich and later of great importance in the evolution of Biochemistry, reduced to a diaminobenzoic acid⁴². In 1872, Griess observed that two of the dinitrobenzoic acids, upon decarboxylation and reduction, yield the same phenylenediamine with a boiling point of 99°C (compound A), and another dinitrobenzoic acid, by the same procedure, yields a phenylenediamine with a boiling point of 140°C (compound F). However, the dinitrobenzoic acid obtained by Cahours could not be decarboxylated and reduced using Griess's procedure⁴³, but in 1874 Casimir Wurster (1854-1913), a student of Victor Meyer, managed to obtain from a barium salt of this acid a phenylenediamine identical to that obtained from the compound pre-

pared by Voit⁴⁴ in 1856, with P.E. 63°C, in 1874, by nitration of (ortho)nitrobenzoic acid. In 1874 Griess obtained two of the dinitrobenzoic acids, B and C (one of which was still unknown, our 2,4-dinitrobenzoic acid, B), which also give rise to decarboxylation and reduction to phenylenediamine⁴⁵ of P. E. 63° C. In 1872, Griess treated 3,4-dinitrobenzoic acid in the same way and obtained ortho-diaminobenzene (compound E). Combining all this data, Griess was able to arrive at the structure of the three diaminobenzenes. Figure 9 illustrates these transformations that lead to the three diaminobenzenes. Manning A. Smith (1915-2012) and William Gower compiled a very enlightening table containing all this information⁴⁶.

The nitration of ortho-nitrobenzoic acid.

This reaction has already been mentioned above, but we will present more detailed information about it here. The nitration of ortho-nitrobenzoic acid with nitric acid in the presence of sulfuric acid forms, according to Griess, four different compounds: styphnic acid (the trinitro-resorcinol of John Stenhouse [1809-1880] and Chevreul), the three isomeric dinitrobenzoic acids of formula $C_7H_4(NO_2)_2O_2$, which he named 1.2.4, 1.2.5 and 1.2.6 (compounds B, C, G in the figure), which are added to the already known compound D, obtained by Cahours in 1846. Compound C was obtained in 1870 by Ferdinand Tiemann (1848-1899) and his collaborator Judson, by oxidation of dinitrotoluene⁴⁷. Compounds A and B were then (1874) still unknown, although the corresponding diaminobenzoic acids were known. All these acids could only be efficiently purified in the form of their barium salts, obtained by reaction with barium carbonate. Griess's description of his experiments shows the empirical, experimental aspect of Organic Chemistry at the time, and since there were no theoretical, or even empirical, explanations for why the reactions occurred (considerations that only slowly began to emerge from that time onwards), Griess also does not explain how styphnic acid is formed from ortho-nitrobenzoic acid in this reaction.

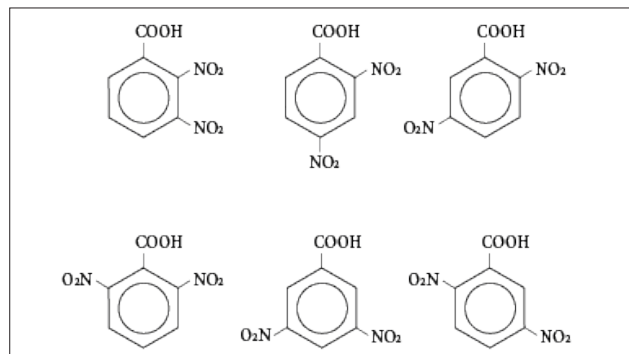


Fig. 10 The six dinitrobenzoic acids

A new derivative of picric acid. The series of picric acid derivatives.

Picric acid (2,4,6-trinitrophenol) was synthesized in 1771 by the Irish chemist Peter Woulfe (1717-1803), and was initially used as a yellow dye. The compound was immediately the subject of much research; for example, in 1848, in a still rudimentary study, Hermann Blumentau (1819-1899), in Trommsdorff's laboratory in Erfurt, observed the decomposition of picric acid into oxalic acid, nitrogen oxides, and carbon dioxide⁴⁸. John Stenhouse (1809-1880) treated picric acid with bromine⁴⁹ and obtained bromopicrin and bromoanil. In 1853, the French chemist Alfred Claude Aimé Girard (1831 Paris-1898 Paris), by selective reduction of picric acid with H_2S , obtained a derivative, picramic acid (2-amino-4,6-dinitrophenol)⁵⁰. It was initially believed that a further reduction of picric acid would not be possible. In 1870, Peter Griess, by modifying the reaction conditions, achieved a new reduction. Powdered picric acid is treated with an aqueous ammonia solution, rapidly introducing a stream of gaseous H_2S , which causes a new reduction, forming 2,4-diamino-6-nitrophenol⁵¹. Like other amino acids, this new compound also has both acidic and basic character, but as a base only with strong acids. As an acid, it forms, for example, barium and silver salts. Griess suggests that his new compound could have use as a dye. With the obtaining of diaminonitrophenol, the series of picric acid derivatives is completed. The first of these derivatives to be obtained was styphnic acid, first obtained in 1808 by Michel Eugène Chevreul (1786-1889), but by nitration of resorcinol, and not from picric acid.

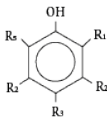
The other two picric acid derivatives were obtained even before Griess's compound, by different reduction processes. Eduard Lautemann (1836 Felsberg/Kassel – 1868 Biebrich/Rhine), a student of Hermann Kolbe in Marburg (1857/1861) and later assistant chemist at the Polytechnic School of Lisbon (1861/1864) and professor at the Medical School of Goa, in Portuguese India, discovered in 1863 the reducing activity of HI on picric acid (the reduction of organic compounds with HI was discovered by Lautemann⁵², and not by Berthelot – the “universal reducing agent of Berthelot” – as is generally read). Lautemann obtained a compound for which he determined the formula $C_6H_2(HO)(NO_2)_3 \cdot (HI)_3$, giving it the name picramine iodide (our picramine)⁵³. Picramine is not a phenolic compound, but it is now known that HI also reduces the OH group, an important fact for the study of carbohydrates. In 1865, Carl Heintzel (1841 Landshut – 1910 Lüneburg), a chemist at the *Gewerbeakademie* in Berlin, treated picric acid with tin and HCl as a reducing agent, and after further treatment with

H₂S, obtained the compound C₆H₂(HO)(NH₃)₃·(HCl)₂, our 2,4,6-triaminophenol, for Heintzel, triaminophenyl chloride⁵⁴. As early as 1867, Heintzel published an extensive article on the work of chemists with picric acid, primarily with its reduction⁵⁵. Thus, “nitrous acid” (HNO₂) or the group NO₂ can be successively replaced by the group NH₂, with the formation of three new compounds:

- aminodinitrophenol
- diaminonitrophenol
- triaminophenol.

The first of these compounds was obtained, as we have seen, by Girard in 1853, the third by Heintzel himself (1865), and the second by Griess in 1870.

Table 1. Picric acid derivatives

	R1	R2	R3	R4	R5	
	NO ₂	H	NO ₂	H	NO ₂	Picric Acid (Woulfe, 1771)
	NH ₂	H	NH ₂	H	NO ₂	Picramic Acid (Girard, 1853)
	NH ₂	H	NH ₂	H	NO ₂	Diaminonitrophenol (Griess, 1870)
	NH ₂	H	NH ₂	H	NH ₂	Triaminophenol (Heintzel, 1865)
	NO ₂	OH	NO ₂	H	NO ₂	Styphnic Acid (Chevreul, 1868)

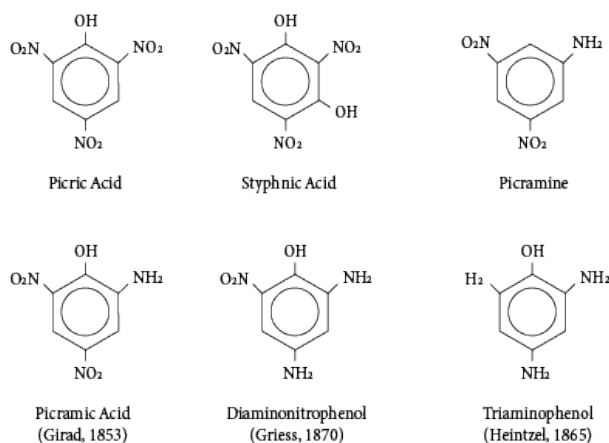


Fig. 11 Picric acid derivatives

Reactions of azo compounds and other nitrogen compounds.

Griess was not a chemist of high theoretical flights, and his range of empirical research was not very varied. His interest lay mainly in nitrogenous organic compounds, and the discovery of diazonium salts followed by that of aromatic azo compounds, among them many dyes, was the raw material for his entire life. Griess was quite jealous of his priority and primacy, as can be seen

in the following example. In 1879, Philipp Weselsky (1828-1889), a professor at the University of Vienna, and his student and collaborator Rudolf Benedikt (1852-1896) announced the discovery of a compound they named ‘azobenzenonaphthylamine’, obtained from the reaction of naphthylamine with diazobenzene⁵⁶. Griess, in an elegant and discreet manner, published another article⁵⁷, communicating that as early as 1864 he had carried out this and other similar reactions, with the publication of an article on the subject⁵⁸, the formation of the compound ‘diazobenzolaminonaphthol’, although it remains unclear whether it was an azo- or a diazo compound. The sulfonic derivative of this compound, with the formula C₁₆H₁₂N₂O₃SO₃, obtained by the reaction of naphthylamine with diazobenzenesulfonic acid, is a dye.

Other nitrogen compounds also sparked his interest. While at the *Royal College*, he studied with Carl Adolph Leibius (1833-1893), Hofmann’s assistant from 1857 to 1859, the action of HCN on methylamine, forming N-methylurea (Leibius was from Württemberg, received his doctorate in Heidelberg in 1857, and emigrated to Australia in 1859, where he was an analyst and, in 1870, chief analyst at the Sydney Mint, becoming a specialist in gold and silver)⁵⁹. Using the same method, Leibius obtained dicyanaminobenzoic acid (C₇H₅(H₂N)₂.2CN) from the reaction of HCN with one of the aminobenzoic acids⁶⁰. From this new derivative of benzoic acid, Griess obtained several products, of which he characterized two, “two new organic bases”⁶¹, to which he attributed the formulas C₇H₆N₂ and C₁₆H₁₈N₄O₆. At that time, the analytical resources available for the structural determination of these benzoic acid derivatives, Griess’s preferred field, were still insufficient: thus, from the first of these bases he concluded only that “it was not the cyanoaniline of [A.W.] Hofmann,” but he intended to obtain it in greater quantity to obtain cyanobenzene and cyanophenol. This analytical difficulty explains Griess’s interest in contributing to the clarification of the positions of the ortho-, meta-, and para- substituents. In 1859, Griess published an article on two new phenol derivatives, dinitrochlorophenol and aminonitrochlorophenol, the latter being obtained from the former⁶². It is difficult to say today what the position of the substituents is on the aromatic ring, since at the time of the synthesis there were no methods capable of determining the exact position, a matter to whose solution Griess himself collaborated, as we saw earlier. In Griess’s opinion, chlorophenol is formed initially, then converted into dinitrochlorophenol.

According to Emil Fischer, in the extensive obituary he wrote in partnership with Hofmann and Bopp⁶³, Griess arrived at his extensive work with azo com-

pounds, which he pursued from 1860 to 1866, in a somewhat convoluted manner, probably starting from Raffaele Piria's (1814-1865) discovery in 1848 of the possibility of replacing an NH_2 group with a hydroxyl group, OH , in the conversion of aspartic acid to malic acid⁶⁴. Along the same lines, A. W. Hofmann and Robert Hunt (1797-1887) obtained phenol from aniline by reaction with nitrous acid (1857). The reaction was repeated and analyzed in 1858 by Augustus Matthiessen (1831-1870), a student of Bunsen, with a reformulation of its representations⁶⁵ (Figure 12).

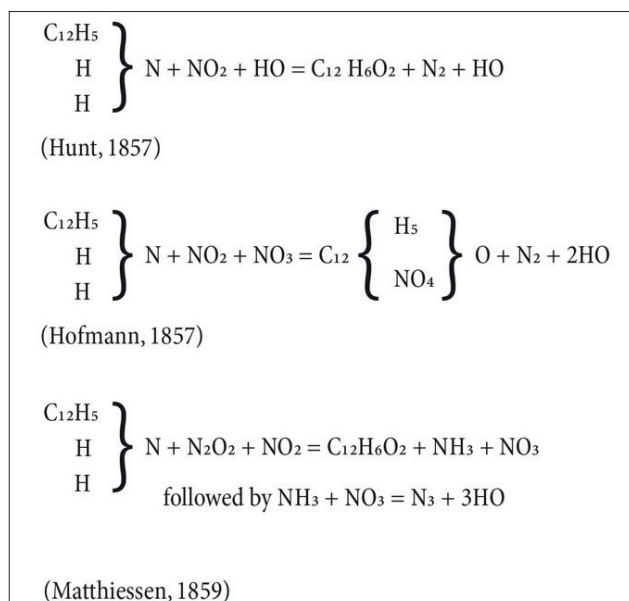
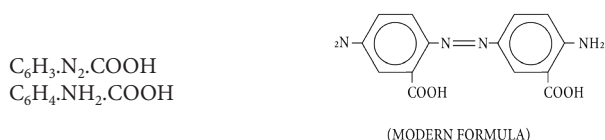


Fig. 12 Some reactions of nitrous acid and aniline

The investigation of the chemistry of azo compounds – their production and properties – did not occur in the systematic way we see it today, because Griess did not bother to explain theoretical aspects, motivation for studying a particular reaction, many details of the experimental part, or difficulties encountered in his articles. The systematization that we know is due to Kekulé, in his “Treatise on Organic Chemistry”⁶⁶.

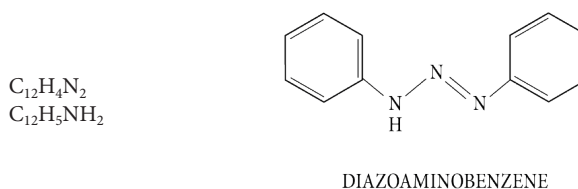
Having studied the nitro- and amino derivatives of phenols, and with aniline still difficult to access, Griess went on to study aminobenzoic acid⁶⁷ (1861), by treatment with nitrous acid he obtained a diazoamino compound to which he assigned the formula:



From anthranilic acid he obtained a more complex compound, to which he assigned the formula:



Only then did he begin to study the behavior of aniline under these conditions, obtaining diazoaminobenzene (1859, published 1862)⁶⁸, the first triazene:



Primarily, this last reaction was of great importance in obtaining azo dyes. The research on compounds grouped as ‘azo compounds’ occupied Griess until 1866, when he began to investigate other nitrogen compounds, generally isolated subjects unrelated to previous research, quite specific. Years later he would return to azo compounds, in articles generically titled “New observations on...”, now including substances such as naphthylamine (which led to Griess’s test for nitrites, discussed above), or benzidine (which led to new dyes, now considered carcinogenic). The most important dye derived from benzidine is Congo Red, developed by Paul Böttiger in 1884 while he was working at *Friedrich Bayer & Co.*, a company that showed no interest in the new dye. Böttiger used it at his father’s textile factory in Łódź and later offered it to *BASF*, which also showed no interest. He then sold the patent to *AGFA*, which made substantial profits from it. Almost nothing is known about Paul Böttiger. Congo Red is still used in biochemical analyses.

In the interval Griess occupied himself with other subjects, of which we will mention two. First, the obtaining of N-alkyl derivatives of aminobenzoic acids (1872), using alkyl iodides and potassium salts, which led to a method of alkylation of amino acids⁶⁹. Thus, starting from meta-aminobenzoic acid and with excess methyl iodide, Griess obtained a betaine, which he represented as [A]; from sulfanilic acid, he obtained the compound which he represented by [B], and from ortho-aminophenol, he obtained the compound represented by [C].

Today we say that we are dealing with a method for obtaining N-aminomethylbenzoic acid, N,N-dimethylaminobenzoic acid, etc. Figure 13 shows our representation of these reactions studied by Griess, who did not have the theoretical knowledge and chemical analysis skills that we have today.

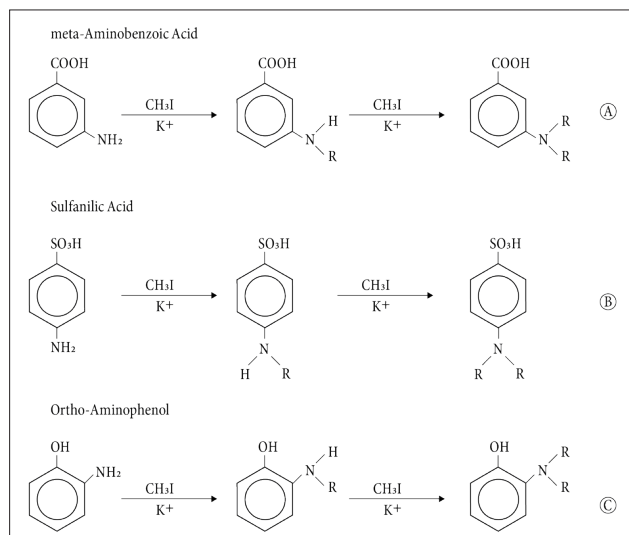


Fig. 13 N-alkyl - and N,N-dialkyl derivatives of aminoaromatic compounds

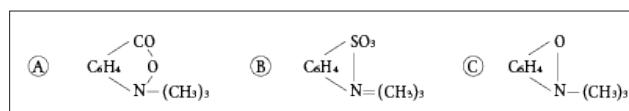
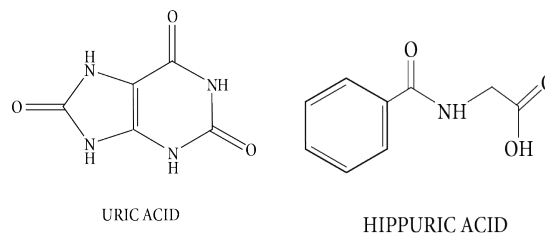


Fig. 14 Fischer's notation for the compounds in Fig. 13 (1894)

Emil Fischer observes that the complexity of the products obtained and the wide variety of possible transformations sometimes led an experienced and patient researcher like Griess down the wrong path, but in the end, the correct results were obtained⁷⁰. This situation is observed in the second group of experiments we wanted to mention, in which Griess worked from 1860 to 1885: the reactions of aminobenzoic acids with cyanide, cyanamide, and urea⁷¹. For example, the reaction of anthranilic acid with the cyano radical in alcoholic solution⁷² leads to a compound to which he assigned the formula $\text{C}_{10}\text{H}_{10}\text{N}_2\text{O}_2$, which with HCl forms $\text{C}_8\text{H}_6\text{N}_2\text{O}_2$, which would be an isomer of azodioxindole, obtained by Baeyer during the synthesis of indigo. To verify if the reaction also occurred with aliphatic amino acids, he reacted glycine with urea⁷³, obtaining a compound of the formula $\text{C}_3\text{H}_6\text{N}_2\text{O}_2$, whose structure he did not determine; had he done so, he would have discovered the synthesis of uric acid, which is now universally attributed to Ivan Horbaczewski (1854-1942)⁷⁴.

A third example (1880) of Griess's reactions involving aromatics is the reaction of isomeric aminophenols (ortho-, para-) with excess methyl iodide CH_3I . The reaction gives rise to betaines, which according to Griess undergo rearrangement to dimethylaminoanisoles⁷⁵. The variant with ethyl iodide does not occur.

Griess also worked with hippuric acid: starting from salts of diazohippuric acid, he obtained new derivatives such as hydroxyhippuric acid ($\text{C}_9\text{H}_9\text{NO}_4$) and iodohippuric acid⁷⁶ ($\text{C}_9\text{H}_8\text{NIO}_3$). Since many of the compounds studied by Griess had complicated structures, he often failed to determine them, identifying new compounds instead through comparison with other compounds and by their physical properties.



CONCLUSION.

Griess was averse to theoretical considerations, but some of his empirical results helped to define certain aspects of the theory, for example, his contribution to clarifying the positions of substituents on the benzene ring.

As an experimental chemist, his case is peculiar: as the head chemist of a large brewery, his experiments in this area, with few exceptions, remained unpublished, as industrial secrets: improvements in fermentation, detection of contaminants, quality control. One of the published works refers to the discovery of choline in hops⁷⁷.

As an experimenter, it could be said that Griess was a 'man of one instrument', but the results he obtained were of enormous importance, both from a scientific point of view (study of diazotization and azo compounds, of aromatic compounds) and from a practical and technological point of view: the use of diazonium salts in organic synthesis, both via azo compounds and through the possibilities of the Sandmeyer reaction. Rarely has a scientific topic been studied with such detail and so intensively as the formation of diazonium salts and azo compounds by Griess.

Interested in practical matters and needing employment, he went to England, as we have seen, at the invitation of A. W. Hofmann to conduct research at the *Royal College of Chemistry*, where he felt comfortable with his German colleagues. Leaving the *College*, he was unable to fully adapt to life in England, beginning with the language (he generally published in German, in journals like the *Berichte* and *Annalen*). He received several invitations to return to Germany, including one from Hein-

rich Caro to work at BASF. He refused them so that his English children would not feel ‘out of place’ in another country, as he himself felt ‘out of place’ in England. Social life intensified with his marriage to Louise Mason, who, however, fell ill and spent years unable to leave the house, eventually becoming bedridden under the care of her husband and housekeeper, until her death in 1886. Griess and his children continued to spend their summer holidays in Bournemouth, on the English Channel, which since 1810 had begun to emerge as a summer resort. During one of these stays in Bournemouth, Peter Griess died of a heart attack on August 30, 1888. He was buried in Burton, next to Louise, in the Mason family tomb.

Griess was a member of the *Royal Society* (1869), received a honorary *PhD* from the University of Munich (1877), and was vice-president of the Chemical Society and of the Society of Industrial Chemistry.



(FIG. 15) Bournemouth, resort where Peter Griess and his family spent their summer holidays. Postcard from 1895, showing the access to the pier (public domain).



(FIG.16) Peter Griess's house in Burton-on-Trent (courtesy Dr. Edwin Yates, University of Liverpool).

AKNOLEDGEMENT

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Research article

Chemethics: Towards an Ethics of Chemical Intervention

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Abstract. The ethics of chemistry occupies an uncomfortable position in the landscape of applied ethics. Even though a significant proportion of the moral challenges generated by contemporary molecular intervention are, at their root, chemical — produced by chemistry's characteristic capacity to decompose, recombine, and produce matter in novel configurations — the living space of ethics of chemistry has been systematically colonised by bioethics, ethics of technology, and public policy discourse. This paper argues that this colonisation reflects a structural misalignment: bioethics is constituted at the level of application rather than at the level of the founding conditions of molecular intervention. To address this gap, the paper proposes the concept of chemethics — an ethics of a type of intervention rather than a disciplinary or professional ethics, applicable wherever chemistry's constitutive agenda operates. Drawing on the genealogy of molecularism, the alchemical dream of total transformation, and the philosophy of chemistry, the paper develops three principles of chemethics — the underdetermination principle, the levels principle, and the limit principle — understood as second-order constraints on any adequate ethics of molecular intervention. The paper concludes that chemethics and bioethics are complementary frameworks: the former addressing the founding conditions of molecular intervention, the latter the application-level questions that arise from them.

Keywords: Chemethics, Ethics of chemistry, Molecularism, Chemical intervention, Bioethics, Phronesis

INTRODUCTION

The ethics of chemistry occupies an uncomfortable position in the landscape of applied ethics. Despite the fact that a significant proportion of the moral challenges generated by modern science and technology are, at their root, chemical — produced by the characteristic capacity of chemistry to decompose, recombine, and produce matter in novel configurations — the field of ethics of chemistry often remains chronically underdeveloped, its living space colonised by bioethics, ethics of technology, corporate ethics, and public policy discourse (Gerontas, 2021). When students are presented in the class with the thalidomide case, they reach for clinical trial regulation. When presented with Agent Orange, they reach for corporate responsibility and government accountabil-

ity. When presented with CRISPR, they reach for bioethics. In each case, the chemical underdetermination at the root of the problem — the fact that a newly synthesised or modified entity's properties cannot be fully specified in advance at the point of synthesis or modification — disappears from view, absorbed into frameworks that were not designed to engage it. This pattern reflects not simply a pedagogical habit, but a structural misalignment between the level at which ethical analysis is conducted and the level at which the intervention operates.

There have been efforts to establish the ethics of chemistry as an autonomous discipline (Schummer & Børsen, 2021), efforts that represent an important step in the right direction. As Joachim Schummer has argued, the ethical specificity of chemistry lies in the fact that its products are not only knowledge but also, and crucially, new material entities that directly transform the world (Schummer, 1997). However, as argued elsewhere, these efforts so far do not yet resolve the foundational problem: they do not demonstrate on conceptual grounds why chemistry, rather than biology or industry or technology, is the primal and decisive protagonist of the moral challenges it addresses — both on the level of the problem and on the level of the remedy (Gerontas, 2021). Without that demonstration, the ethics of chemistry remains a collection of case studies that other ethical frameworks can always claim with equal or greater institutional confidence. The current cultural dominance of molecularism has deepened this problem — the framework, operative simultaneously in scientific self-understanding, popular culture, policy, and commercial practice, in which life is conceived as constituted by manipulable molecular units, and in which knowledge of life is intrinsically linked to the capacity to intervene in and redesign it; a cultural myth, in Mary Midgley's sense, that molecular biology's success made available and that the broader culture proceeded to inhabit (Gerontas, 2026). It is this “cultural” molecularism that has consolidated bioethics as the default primary framework for the moral challenges of molecular intervention on living matter, by making the interventive orientation appear as the natural direction of biological progress rather than a historically contingent development rooted in chemistry's constitutive agenda.

Molecularism, as the term is used here, names an epistemic and cultural framework rather than a scientific theory or a synonym for molecular biology: the orientation in which living systems become intelligible primarily through their molecular constitution and governable through molecular intervention, so that understanding a system is increasingly identified with the capacity to analyse, manipulate, and redesign it at the molecular level.

This paper proposes a conceptual foundation that complements these existing frameworks by operating at a different level of analysis. The ethics adequate to molecular intervention — to CRISPR, synthetic biology, AI-driven drug design, genomic enhancement, and the full range of practices in which molecularism has become practice — requires a framework constituted not at the level of any particular discipline or profession, but at the level of the act of chemical transformation itself: the manipulation of matter at the level of its constitutive transformability. The social dimensions of technoscientific intervention have been extensively explored in science and technology studies (Jasanoff, 2016; Feenberg, 2002), and the question of values embedded in technological design has been a central concern of the philosophy of technology (Winner, 1980). What has not been proposed is a framework that grounds the ethics of molecular intervention specifically in the constitutive features of chemical transformation as such. This paper proposes the term *chemethics* to name that framework. Chemethics is not the ethics of the chemistry profession, nor the ethics of chemical weapons or industrial pollution — these are disciplinary or application-specific ethics — but an ethics of a type of intervention, applicable wherever the chemical agenda operates: in molecular biology as much as in synthetic chemistry, in pharmaceutical development as much as in materials science, in AI-driven biology as much as in the laboratory.

The argument proceeds in four steps. Section 1 diagnoses the structural limitation of bioethics as currently practised in the domain of molecular intervention, showing that it operates within the molecularist framework it should be interrogating rather than examining the epistemic and cultural assumptions that make molecular intervention conceivable. The issue is not the absence of reflection within bioethics, but the default framing of problems at the level of application rather than at the level of constitution. Section 2 argues that if biology's interventive orientation is derived from chemistry's constitutive agenda — as established in prior work (Gerontas, 2026) — then the ethics corresponding to this orientation must be grounded there, and develops the concept of chemethics accordingly. Section 3 traces the deeper genealogy of the interventive orientation to the alchemical dream of total transformation, arguing that molecularism's most characteristic ambitions — the perfection of the body, the overcoming of mortality, the redesign of life — can be understood as structural continuities of alchemy rather than novelties of modern science, and that chemethics must engage this genealogy if it is to be adequate to the depth of the orientation it is addressing. Section 4 proposes three principles of chem-

ethics — the underdetermination principle, the levels principle, and the limit principle — understood not as first-order norms but as second-order constraints: conditions of adequacy that any ethics of molecular intervention must satisfy if it is to engage the phenomena at the appropriate level. The paper concludes by arguing that chemethics and bioethics are complementary frameworks operating at different but mutually necessary levels of analysis — the former addressing the founding conditions of molecular intervention, the latter the application-level questions that arise from them.

Because the word *level* will do a great deal of work in what follows, three senses of it should be separated at the outset. There is the *level of intervention* — for the cases at issue, the molecular level at which matter is manipulated. There are the *levels of biological organisation* — molecule, cell, tissue, organism, population, ecosystem — across which the effects of a molecular intervention propagate. And there is the *level of ethical analysis* — the distinction between an ethics constituted at the level of an intervention's application and one constituted at the level of its founding conditions. When this paper argues that chemethics operates at the level of founding conditions *rather than* applications, it speaks in the third sense; when it proposes a levels principle, it speaks in the second. The apparent tension between the two dissolves once the senses are kept apart: the framework is *situated* by the third sense, and it *directs attention*, through its second principle, to the second.

1. BIOETHICS AND THE FOUNDING CONDITIONS OF MOLECULAR INTERVENTION

The resources that bioethics brings to the moral challenges of contemporary science and medicine are real and substantial. Over half a century, the field has developed a sophisticated apparatus for ethical reasoning in clinical and research contexts: the four-principles framework of autonomy, beneficence, non-maleficence, and justice that has become the discipline's most influential organising structure (Beauchamp & Childress, 2019); extensive literatures on informed consent, research ethics, organ transplantation, end-of-life care, and reproductive technology; and institutional mechanisms — ethics committees, regulatory frameworks, review boards — that have transformed the conditions under which medical and biological research is conducted (Rothman, 1991). This apparatus has done important work, and nothing in what follows is a rejection of it. The argument here is a bit more specific: that bioethics as currently constituted operates at the level of application rather than constitution, and that

this constitutive feature means it is not engaged at the level of analysis that would address the founding conditions of molecular intervention — the conditions that make the applications conceivable in the first place.

The historical roots of this limitation are instructive. Bioethics emerged as a recognisable discipline in the 1960s and 1970s, shaped by a specific set of founding cases: the Tuskegee syphilis study, the development of intensive care medicine and organ transplantation, the thalidomide tragedy, and the first experiments in recombinant DNA technology. What these cases had in common was not that they raised questions about the nature of the interventions involved, but that they raised questions about the governance of interventions whose nature was already understood — or assumed to be understood. The Tuskegee study raised questions about the ethics of withholding treatment and the conditions of informed consent; organ transplantation raised questions about the allocation of scarce medical resources and the definition of death; the thalidomide case raised questions about the regulation of clinical trials and the responsibilities of pharmaceutical companies. In each instance, bioethics was constituted as a discipline for assessing the consequences and governing the practice of interventions that were taken as given. The level of analysis was the application; the intervention itself — what kind of thing was being done, what assumptions it carried, what it presupposed about the nature of the entities it was acting on — was outside the frame.

This constitutive orientation was not a failure of vision but a reflection of the cases that founded the discipline. When molecular biology subsequently generated applications — recombinant DNA technology, gene therapy, genomic sequencing, and eventually the CRISPR-Cas9 system — bioethics was the available framework, and it applied its characteristic approach: assess the consequences, specify the principles, regulate the practice. The result has been an extensive and valuable literature on the ethics of genetic intervention. Nevertheless, a literature realised, at almost every point, at the level of application rather than constitution. The debate over germline editing — concentrated by the announcement of the first CRISPR-edited human births in 2018 and the international call for a moratorium that followed (Lander et al., 2019) — illustrates the pattern with particular clarity. The debate addresses questions of safety, consent, equity of access, the distinction between therapeutic and enhancement uses, and the governance of heritable modification. All these are genuine and important questions. What the debate does not ask — or asks only at its margins — is whether the molecular level is the appropriate level at which to assess what is being done or not.

The question matters because the relationship between genotype and phenotype is massively underdetermined. The traits most discussed in fashionable enhancement contexts — cognitive capacity, physical performance, disease resistance — are polygenic, developmentally sensitive, and shaped by environmental interactions that current molecular biology cannot fully specify. An intervention that is completely characterised at the molecular level may generate effects at the cellular, developmental, organismal, and population levels that the molecular specification neither predicts nor anticipates. Importantly, this is not a contingent gap in current knowledge that further research will fill; it is a structural feature of the kind of intervention that CRISPR performs — a chemical intervention in a living system organised at multiple levels, each with its own dynamics and regularities that the molecular level does not determine. Bioethics, operating within the molecularist framework that treats the molecular level as the salient level of *organisation*, tends to assess the intervention at the organisational level where it is performed rather than tracking it across the levels where its consequences propagate — even as, in the third sense distinguished above, it remains constituted at the level of *application*. The two are consistent: a framework built to govern given applications inherits from molecularism the assumption that the molecular level is where the relevant description lies.

It should be said plainly, since a reader might reasonably ask, that bioethics does address several of these organisational levels in practice — ecological risk, population effects, and long-term consequences all appear in its literatures. The claim is not that it ignores the higher levels but that it engages them at the level of application: as consequences to be assessed once an intervention is given, rather than as founding conditions bearing on what kind of ethics the intervention requires in the first place. The difference is not the range of levels surveyed but the level at which the framework is constituted.

The thalidomide case makes the structural point in its starkest form — and also illustrates why the point consistently eludes bioethical analysis. Thalidomide was synthesised, its sedative and anti-nausea properties were established, and its catastrophic teratogenic effects were not and could not have been fully anticipated at the point of synthesis: the chemical underdetermination of a novel molecular entity meant that its full range of biological effects was unknowable in advance. The moral problem originated in the act of chemical intervention itself — in the production of an entity whose properties were underdetermined in advance — yet bioethics engaged it only downstream, as a matter of clinical-trial regulation, pharmacovigilance, and the responsibilities of the phar-

maceutical industry (Ruthenberg, 2021). The shift is not from one organisational level to another but from the founding conditions of the intervention to its governance: the underdetermination that generated the tragedy was left unaddressed *as such*, because the framework that took up the case was built to govern applications, not to interrogate the conditions that make them possible. These regulatory responses were necessary and valuable of course. But they addressed the consequences of chemical underdetermination without engaging the underdetermination itself as a structural feature of the type of intervention that produced the problem. The question that bioethics did not ask — because it was not constituted to ask it — was what kind of ethics is adequate to an intervention that characteristically produces entities whose properties cannot be fully specified in advance.

That question is the founding question of the chemethics proposed here. It does not replace the bioethical questions about thalidomide, CRISPR, or synthetic biology. It precedes them — establishing the level at which the ethical analysis must be grounded before the application-level questions can be adequately addressed. Bioethics, for all its sophistication, addresses consequences while leaving their constitutive conditions unexamined. Chemethics proposes to examine them.

2. CHEMISTRY'S CONSTITUTIVE AGENDA AND THE ETHICS IT IMPLIES

The existing literature on the ethics of chemistry has established two important foundations for the argument developed here. Schummer's 2001 paper in *HYLE* — a quite systematic analysis of moral issues arising from chemical synthesis — demonstrated that chemical synthesis is not morally neutral: unlike other branches of science, chemistry's products are not only ideas but also new material entities that change the world, and the chemist who produces them incurs ethical responsibilities that cannot be reduced to those of any other scientific practitioner (Schummer, 2001). Mehlich et al. (2017), surveying the ethical and social dimensions of chemistry more broadly, showed that the chemist faces challenges belonging simultaneously to science ethics, research ethics, engineering ethics, and professional ethics — a mapping of ethical territory that confirmed both the genuine distinctiveness of chemistry's ethical situation and the difficulty of housing it within any single existing framework. Neither contribution, however, proposes an ethics constituted at the level of the intervention type rather than at the level of the profession or discipline. Schummer's ethics of chemical synthesis asks what moral obli-

gations the chemist incurs by performing synthesis, stressing that responsibility does not necessarily imply moral judgement; Mehlich et al. ask what ethical challenges the chemistry profession faces; and both remain within the structure of professional or disciplinary ethics. What neither address is the question this paper takes as its starting point: what kind of ethics is adequate to the act of chemical transformation itself, independently of the institutional home in which it is performed and the professional identity performing it?

The difference from Schummer is therefore one of logical order rather than of vocabulary. Schummer specifies first-order moral obligations that the chemist incurs in performing synthesis; chemethics specifies second-order conditions of adequacy that any ethics of molecular intervention must satisfy, whoever performs it and under whatever obligations. The two are not rivals: Schummer's obligations are among the first-order norms that a framework meeting the chemethical conditions would go on to articulate.

The answer proposed here begins with the account of chemistry's constitutive agenda developed in prior work (Gerontas, 2026). Chemistry is not only a science of the molecular constitution of matter; it is, distinctively among the natural sciences, a productive science — one in which understanding a material system is inseparable from the capacity to decompose, recombine, and produce it. Bensaude-Vincent and Simon (2008) identify this productive orientation as chemistry's constitutive agenda: the identification of knowing with transforming, of comprehension with the capacity to synthesise. This agenda is not an accidental feature of chemistry's historical development; it is constitutive of what chemistry is as a form of inquiry. A science that could only describe and explain matter, but not intervene in and produce it, would not be chemistry in any recognisable sense (most probably, it would be a branch of physics).

The ethically significant consequence of this constitutive agenda is Schummer's underdetermination point, now seen in its full structural scope. Because chemistry's primary activity is the production of novel material entities — entities that did not previously exist — the properties of its products cannot be fully specified in advance of their production. This is not a contingent epistemic limitation, but a structural feature of productive knowledge: the entity produced by chemical intervention is genuinely new, and its full range of properties, interactions, and effects in complex systems can only be discovered after it is free to interact with the world. The ethics corresponding to this situation cannot be a simple application of pre-existing norms to known outcomes. It must be an ethics of the encounter with the genuinely

new — an ethics of radical novelty as a structural condition rather than an exceptional circumstance.

This structural feature — productive novelty as a constitutive condition rather than an occasional complication — is what bioethics is not constituted to engage, and what an ethics of the intervention type must take as its foundational datum. It is also what makes it necessary to speak of an ethics of chemical intervention rather than an ethics of the chemistry profession. When the productive orientation of chemistry migrates — as this paper's prior work has argued, molecularism has caused it to do so — into molecular biology, synthetic biology, pharmaceutical development, and AI-driven biological research, the ethics corresponding to it migrates too. The chemist performing a synthesis in a laboratory, the molecular biologist editing a genome, the synthetic biologist constructing a novel metabolic pathway, and the AI system proposing novel drug candidates are all performing variations of the same fundamental type of intervention: the manipulation of matter at the level of its constitutive transformability, generating entities whose full properties are underdetermined in advance. An ethics adequate to this situation must be constituted at the level of the intervention type — chemethics — rather than at the level of any of the disciplines or professions in which the intervention is housed.

Chemethics, as here proposed, is therefore defined by three features that distinguish it from the existing frameworks reviewed above. First, it is an ethics of intervention rather than a disciplinary or professional ethics: its scope is determined by what is being done, not by who is doing it or within what institutional framework. Second, its foundational datum is productive novelty — the structural underdetermination of the properties of newly created entities — rather than the application of known procedures to known outcomes. Third, it operates at the level of the founding conditions of molecular intervention rather than its applications: it asks what assumptions about life and matter the intervention carries before asking what should be done with the results. In this sense, chemethics does not replace the existing ethics of chemistry literature or the bioethical frameworks that address specific applications; it proposes the level of analysis at which both must be situated if they are to fully engage with the phenomena.

3. THE ALCHEMICAL DREAM: STRUCTURAL CONTINUITY

The argument of this section is genealogical, and the sense of genealogy must be stated precisely, because it is easily mistaken. It is not the claim that modern chemis-

try inherited the theories, the cosmology, or the mystical apparatus of alchemy; the historiography that has dismantled that picture is by now decisive (Principe, 2013), and nothing here depends on reopening it. Nor is it the claim that a promethean ambition animates molecular intervention *as such*: the overwhelming majority of chemical and molecular work is patient, local, and modest in its aims, and no genealogy is required to understand it. The claim is narrower and structural. What alchemy first made explicit — and what modern chemistry did not so much refute as absorb — is an orientation in which the knowledge of matter is inseparable from its transformation, and in which that transformation is imagined, *at its horizon*, as the overcoming of the given limits of material and bodily existence. It is this orientation — not any doctrine, and not any agent's intention — that constitutes the continuity at issue; and it becomes ethically consequential only where intervention reaches toward that horizon.

That the continuity runs *through* the constitution of modern chemistry rather than around it is not an external imposition on the discipline but something its founders themselves recognised. Reflecting, in the third of his *Chemische Briefe*, on the alchemical search for the philosopher's stone, Justus von Liebig judged the idea to have been indispensable to the science that superseded it: no finer notion, he wrote, could have been conceived to inspire the minds of men, for without it chemistry would not have become what it is, and it was only in the effort to discover that no such thing existed that it became necessary to ransack and analyse every substance known on earth (Liebig, 1851). Liebig's point is not nostalgic. He identifies the philosopher's stone as the organising aim whose pursuit produced the systematic interrogation of matter — the productive orientation itself — even as its literal object proved to be nothing. The dream was constitutive of the method precisely in being unrealisable.

Read through Mary Midgley's conception of myth (Midgley, 2003), this is exactly what a myth in her sense does. A myth is not a false belief held by individuals but an imaginative background — a pattern of pictures and aspirations that organises inquiry and directs practice below the level of explicit doctrine. To identify the philosopher's stone, and its molecular heirs, as a myth in this sense is therefore not to attribute a fantasy to anyone, nor to impute a hidden intention to the molecular biologist or the synthetic chemist. It is to name the imaginative structure within which the interventive orientation acquires its characteristic horizon — the perfection of matter, the perfection of the body, the overcoming of mortality — and against which the question of limits does not spontaneously arise.

Alchemy was organised around an ambition: the

transformation of matter to its highest potential. The philosopher's stone, the elixir of life, and the homunculus are not three separate projects but three expressions of a single structural logic — the complete mastery of material transformation, applied to metal, to the body, and to life itself. What is philosophically significant for the present argument is that this logic did not disappear when alchemy's cosmological and mystical apparatus became untenable. It migrated. The Baconian programme — knowledge as power over nature, science as the systematic improvement of the human condition through mastery of material processes — gave the ambition an epistemological and institutional home without its metaphysical scaffolding. What survived was the productive orientation, the identification of knowing with transforming, and the aspiration toward overcoming the given conditions of material existence.

Paracelsus (1493–1541) is the relevant pivot figure here, though not as the last alchemist — alchemical practice remained intellectually active well into the seventeenth century and beyond, as recent scholarship has thoroughly documented (Principe, 2013) — but as the figure who most systematically applied the alchemical logic of perfection-through-transformation to medicine as a programme. In Paracelsus' framework, the physician is not primarily a diagnostician but a chemist: disease is a chemical imbalance, health is the outcome of successful chemical transformation, and the body is an object of productive intervention. This is one of the earliest moments at which the ethics of chemical intervention in living matter becomes a practical rather than merely a theoretical question — one of the first chemethical moments, in the sense this paper has given that term.

The structural continuity with the molecularism of today is precise. The aspiration to edit the genome — to correct inherited imperfections, to enhance desired traits — is the philosopher's stone applied to the biological substrate. The aspiration to overcome ageing is the elixir of life in molecular form. The aspiration of synthetic biology to construct living systems from designed components is the homunculus made technically feasible. To call these structural continuities rather than poetic analogies is to claim that the same organising myth — that material transformation, pursued to its limit, can overcome the given conditions of biological existence — is operative in both, regardless of the radical difference in technical means. The claim is not that every genome edit or synthetic construct is driven by such an aspiration, but that where the interventive orientation reaches toward that horizon, it reactivates a pattern that alchemy was the first to make explicit.¹

¹ It is worth noting that this structural and imaginative relation has not been lost to the molecular biology community. It is either openly

This genealogy matters for chemethics because it specifies the depth at which the ethics of chemical intervention must operate. The three principles proposed in the following section are not merely technical constraints on molecular research; they answer to an orientation that has long organised the relationship between chemical knowledge and the human body, and, by recent extension, living matter in general. The limit principle in particular responds to a characteristic feature of that orientation: within an imaginative horizon organised around transformation, limits present themselves by default as provisional obstacles rather than as features of the phenomena that might warrant ethical engagement. It is important to be clear about the role the genealogy plays here. It does not *ground* the limit principle — as the next section argues, that principle draws its justification from the structural features of multi-level, underdetermined intervention, not from the genealogy itself. The genealogy's role is diagnostic: it explains why the question of limits is so readily suppressed, and therefore why an ethics of molecular intervention must take deliberate steps to restore it.

4. THE PRINCIPLES OF CHEMETHICS

The three principles proposed in this section are not first-order norms — they do not specify what ought to be done in specific cases of molecular intervention. They are second-order constraints: conditions of adequacy that any ethics of molecular intervention must satisfy if it is to engage the phenomena at the appropriate level. This is a meta-ethical move, and it requires brief justification before the principles themselves are stated.

The is/ought problem — the impossibility of deriving normative conclusions from purely descriptive premises — is a genuine constraint on ethical argument. However, the principles of chemethics are not derived from facts about chemical intervention; they are derived from the structural features of productive knowledge that any adequate ethical framework must take into account. The analogy is with the conditions of possibility argument in Kantian ethics: before issuing categorical imperatives, the Critique of Practical Reason establishes what any viable moral framework must presuppose. Chemethics operates analogously at a domain-specific

level. The underdetermination of novel entities, the multi-level organisation of living systems, and the alchemical genealogy of the interventive orientation are not facts from which norms follow, but structural features of the phenomena that rule out certain ethical approaches as constitutively inadequate. A framework that ignores them is not engaged with the phenomena it claims to address.

The three principles follow from the three structural features identified in the preceding sections.

The underdetermination principle derives from the constitutive agenda of chemistry as analysed by Schummer (1997, 2001) and Bensaude-Vincent and Simon (2008). Because chemical intervention characteristically produces entities whose properties cannot be fully specified in advance, any ethics of molecular intervention that operates only based on anticipated outcomes is structurally inadequate to the phenomena. This principle does not prohibit intervention with uncertain consequences — it prohibits treating uncertainty as a merely contingent feature of current knowledge rather than a structural feature of productive novelty. Its normative consequence is that any ethics of molecular intervention must build epistemic humility into its foundations, not treat it as a residual caveat. It rules out consequentialist frameworks that require full specification of outcomes in advance, and it rules out rule-following frameworks that apply pre-existing norms to genuinely novel entities as if novelty were merely a matter of complexity. What it points toward is an ethics of judgement under conditions of irreducible uncertainty — *phronesis* in the Aristotelian sense, to which we return below. A concrete illustration: the development of CRISPR base editors, initially presented as a more precise alternative to standard CRISPR-Cas9, subsequently revealed unanticipated cytosine deamination at non-target sites and RNA off-target effects that the initial molecular specification did not predict (Zuo et al., 2019; Gruenewald et al., 2019). The underdetermination was not resolved by increased precision — it was reproduced instead at a finer level of molecular resolution.

This underdetermination must be distinguished from two principles that share its name or its vicinity. It is not the Duhem–Quine underdetermination of theory by evidence, which concerns the relation between a body of data and the theories compatible with it — an epistemic relation internal to representation. Nor is it the indeterminacy of quantum mechanics, which concerns the limits of the simultaneous measurement of conjugate variables in a physical system. Chemethical underdetermination is neither representational nor measurement-theoretic but *productive*: the Duhem–Quine thesis says that our theories are underdetermined by what we can

acknowledged, as is most visible in the extreme case of Rifkin's *Algeny* (1983), or reformulated in contemporary vocabulary — as in recent aspirations to “solve disease” in AI-driven biomedical research (Lowe, 2025). In the latter case, the potential for conflict becomes apparent: while AI is envisaged as a kind of philosopher's stone, a sufficiently capable system may ultimately come to occupy the role of the Alchemist itself.

observe, whereas the underdetermination principle says that the products of chemical intervention are underdetermined by the specifications under which they are made. The first is a limit on knowing; the second is a feature of making.

The levels principle presupposes, and should be stated as presupposing, an anti-reductionist account of the organisation of matter: the thesis, developed in different idioms by Wimsatt (1994) and Dupré (2012), that the higher levels of biological organisation possess properties and causal capacities not exhausted by, and not derivable from, the molecular description of their constituents. Without this commitment the principle would collapse — if everything true of the organism were in principle readable from its molecules, assessment at the molecular level would suffice. Grounded in this anti-reductionism, the principle draws on the account of biological hierarchy discussed elsewhere (Gerontas, 2026) and on the Dobzhansky argument about the levels of biological organisation (Dobzhansky, 1964). Intervention at the molecular level generates effects at the cellular, developmental, organismal, ecological, and evolutionary levels that cannot be read off from the molecular specification. Any ethics that assesses only at the level of intervention is therefore constitutively blind to a necessary dimension of the phenomena. This is not a call for complete knowledge before action — which would prohibit all intervention — but a structural requirement that ethical assessment operate across levels rather than only at the level where the intervention is performed. It is the structural reason why bioethics operating within the molecularist framework systematically tends to underestimate consequences: it is constituted at the level of application, while the effects propagate across levels that the molecularist framework renders secondary. The introduction of Bt crops — genetically modified to express a bacterial insecticidal protein — illustrates the point. At the molecular level the modification is well characterised; at the ecological level, widespread adoption has generated selection pressure for resistance in target pest populations, altered non-target insect communities, and produced cascading effects on predator-prey relationships that the molecular specification did not (and could not) anticipate.

The limit principle has been the most exposed to misunderstanding, and it is worth reconstructing its derivation so that it rests on the two principles already stated rather than on the genealogy alone. It does not assert that there are fixed ontological boundaries in matter — a sharp line, at the molecular level, between the living and the non-living, or between the natural and the artificial. No such boundary is needed, and the philosophy of chemistry gives little reason to expect one.

What the principle asserts is an *asymmetry of justification* that follows directly from the levels and underdetermination principles. If the effects of a molecular intervention propagate across levels that the molecular specification cannot anticipate, and if the properties of the entities it produces are underdetermined in advance, then a given biological limit — the finitude of the lifespan, the boundaries of a species, the stability of a developmental programme — cannot be known to be a merely technical obstacle from the molecular level at which its removal is proposed. Its status as obstacle or as constitutive feature is settled, if at all, only at the higher levels whose functions the molecular framework renders secondary. The burden of justification therefore falls, structurally and not merely as a matter of prudence, on whoever would treat the limit as provisional: the claim that a limit is *merely* an obstacle is a claim about the very levels the intervention cannot see, and it is that claim, not its denial, that requires demonstration.

The alchemical genealogy enters here not as the ground of the principle but as the diagnosis of why the question it raises is so rarely asked: within an imaginative horizon organised around transformation, limits present themselves as obstacles by default. The genealogy explains the blind spot; the levels and underdetermination principles justify its correction. This is why the principle is reflexive rather than conservative. Current research programmes in biological ageing — aimed at extending the human lifespan by intervening in the molecular mechanisms of senescence — illustrate its application. The limit principle does not say such research is wrong, and it takes no position on whether ageing ought to be overcome; it says only that the assumption driving it — that ageing is a pathology to be corrected rather than an evolved feature of organisms organised at multiple levels — must be stated as a claim and defended, not inherited as the natural direction of progress. Ageing is entangled with developmental programmes, ecological roles, and population-level dynamics whose elimination at the molecular level cannot be assessed without engaging the levels at which its functions operate.

Taken together, the three principles identify the natural philosophical home of chemethics as phronesis — Aristotelian practical wisdom. If the properties of novel entities cannot be fully specified in advance (underdetermination), if effects propagate across levels that cannot be fully anticipated (levels), and if the organising fantasy systematically suppresses the question of limits (limit), then the ethics adequate to molecular intervention cannot be a rule-following ethics or a calculation of specified outcomes. It must be an ethics of perception and judgement — the capacity to discern what a genuinely

novel situation requires, across multiple dimensions, without the guidance of pre-existing rules that were not designed for it. This is precisely what Aristotle meant by *phronesis*: not the mechanical application of universal principles to particular cases, but the cultivated capacity to perceive the morally relevant features of a situation and respond to them appropriately (Aristotle, NE VI). Chemethics does not generate a new set of rules for molecular intervention; it specifies the kind of ethical agency — practically wise, attentive to levels, responsive to novelty, and reflective about its own organising assumptions — that molecular intervention requires.

It may be objected that ethical traditions adequate to deep uncertainty already exist, and this is true. Aristotelian practical wisdom, to which the three principles jointly point, is precisely an ethics of judgement where rules underdetermine action; the precautionary principle, arising in the same technologically advanced societies that produced the interventions at issue, codifies restraint under uncertainty; and several indigenous traditions — the injunction, on Turtle Island, to weigh a decision against its consequences seven generations hence — were framed for exactly the situation in which effects outrun foresight. Chemethics does not compete with these; it draws on the first explicitly and stands in evident kinship with the others. What it adds is not a new attitude toward uncertainty but the identification of a constitutive *object* that these traditions address contingently and chemethics addresses as such — the structural underdetermination, cross-level propagation, and limit-suppression specific to productive intervention in matter. The precautionary principle tells us to be cautious; the underdetermination principle tells us why caution is here a structural requirement and not a policy choice.

CONCLUSION

The argument of this paper has moved through four steps. It began with a diagnosis: that bioethics as currently constituted is not engaged at the level of analysis that would address the founding conditions of molecular intervention, and that this constitutive limitation is not accidental but structural — the consequence of a discipline formed to govern the applications of interventions whose nature it did not need to interrogate. It then proposed a genealogical grounding: that the ethics adequate to molecular intervention must be constituted at the level of chemistry's constitutive agenda — the productive orientation in which knowing is inseparable from transforming — because it is from that agenda that molecular biology's interventive orientation derives. It traced that

agenda further, to the alchemical dream of total transformation that modern chemistry institutionalised rather than dissolved, and whose current form is molecularism. And it proposed three principles — underdetermination, levels, and limit — understood not as first-order norms but as conditions of adequacy for any ethics that takes the structural features of molecular intervention seriously.

The concept that organises these four steps is chemethics: an ethics of the intervention type rather than the discipline, constituted at the level of the act of chemical transformation itself, applicable wherever the chemical agenda operates. The relationship between chemethics and bioethics is one of complementarity rather than competition. Bioethics addresses the application-level questions that arise from molecular intervention — what should be done, by whom, under what conditions of consent and governance — and does so with considerable sophistication. Chemethics addresses a different level: the founding conditions of the intervention itself, the structural features of productive novelty that make the applications conceivable, and the genealogical assumptions that any application-level ethics inherits, whether or not it examines them. Where the two frameworks work in conjunction — chemethics establishing the level of analysis at which the constitutive features of the intervention are visible, bioethics addressing the application-level questions that arise from them — the combined framework is more adequate to the phenomena than either alone. The ethics of molecular biology, synthetic biology, and AI-driven intervention in living systems is, at its foundations, a chapter in the ethics of chemistry. And the ethics of chemistry is, at its deepest level, an engagement with the alchemical dream of total transformation that five centuries of productive science have elaborated, refined, and made technically feasible without ever subjecting to systematic ethical examination.

The urgency of this examination is not abstract. CRISPR-based interventions are moving from laboratory to clinic. Synthetic biology is beginning to reshape agriculture, materials science, and pharmaceutical production. AI systems trained on molecular data are proposing novel compounds and redesigning biological pathways at a pace that outstrips the capacity of application-level ethics to assess them. In each case, the molecularist framework supplies the operating assumptions and metaphors — life as molecular information, biology as engineering, progress as increasing molecular resolution and control — and the alchemical dream supplies the horizon: the overcoming of given biological limits as the natural direction of the enterprise. Chemethics does not oppose this enterprise. It insists, however, that the

framework and the dream be made visible, examined, and justified — that the burden of argument fall where it belongs, on those who treat the given conditions of biological existence as merely provisional obstacles, rather than on those who ask what it means to intervene in living systems whose organisation exceeds the molecular level at which the intervention is performed.

Bioethics will continue to do important work at the level for which it was constituted. The closing formulation is therefore not a dismissal but an invitation to collaboration at a deeper level of analysis: current bioethics is chemethics that has not yet recognised its own genealogy. The recognition, when it comes, will not replace bioethics — it will ground it.

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Research Article

DNA as a Language: A Linguistic Comparison with Human Spoken and Written Systems

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Abstract. The traditional metaphor of DNA as a language is examined through systematic analysis, using formal linguistic categories rather than relying on heuristic analogies. By exploring aspects of phonology, morphology, syntax, semantics, pragmatics, and diachrony, the study investigates the structural parallels between genetic sequences and human spoken and written languages, while also identifying the fundamental limitations of this comparison. The analysis indicates that DNA displays distinct symbolic units, a combinatorial organization, strict ordering constraints, context dependence, and historical evolution. These characteristics resemble written language more closely than they do speech. However, DNA fundamentally differs from human language in several ways: it lacks intentionality, reference, generativity, and representational meaning. Genetic “meaning” is defined by its operational and causal roles, focusing on biochemical functions rather than semantic interpretations. This study emphasizes that DNA should not be viewed as a literal language but rather as a system of symbols grounded in chemistry. The comparison with linguistics is most useful for establishing distinctions rather than for expanding metaphorical interpretations.

Keywords: DNA language, morphemes, codons, gene expression, linguistics

The metaphor of DNA as a “language” has circulated in molecular biology since the mid-twentieth century, often as a heuristic for explaining how genetic sequences encode and transmit information. When examined through the formal methodology of linguistics rather than metaphor alone, this comparison becomes both more precise and more constrained. Linguistics offers a multi-level analytical framework—encompassing phonology, morphology, syntax, semantics, pragmatics, and diachrony—that allows for systematic comparison between symbol systems. Applying these categories to DNA clarifies where the analogy with human spoken and written languages is structurally informative and where it breaks down in principle.

UNITS OF EXPRESSION: PHONOLOGY AND ORTHOGRAPHY

Human spoken languages are organized around phonology: a finite inventory of discrete, contrastive sound units (phonemes) that are meaningless in isolation but meaningful in combination [1]. Written language introduces an orthographic layer, in which visual symbols represent phonological units or morphemes depending on the writing system [2].

DNA lacks phonology in the acoustic sense, but it does possess a discrete symbolic inventory analogous to an orthography. The four nucleotide bases—adenine, thymine, cytosine, and guanine—form a finite set of contrastive units whose sequence determines downstream effects in protein synthesis [3]. Unlike phonemes, however, nucleotides are not abstract cognitive categories; they are chemically instantiated entities whose physical properties are inseparable from their informational role [4].

In this respect, DNA more closely resembles written language than speech, yet the analogy remains partial. Orthographic symbols conventionally stand for sounds or meanings external to the script, whereas DNA bases directly participate in the biochemical processes that “read” them. The symbol and its material realization cannot be cleanly separated, a point emphasized in discussions of the symbol–matter relationship in biology [5]. For instance, in the human β -globin gene, a single nucleotide substitution (A→T) changes the DNA codon from GAG to GTG. This alteration results in the incorporation of a different amino acid—valine instead of glutamic acid—into the hemoglobin protein, leading to the development of sickle-cell anemia [6]. This example illustrates that, much as changing a single letter in a word can significantly alter the “meaning” of a word, altering a single nucleotide can significantly change the “meaning” in the language of genetics.

MORPHOLOGY: FROM MORPHEMES TO CODONS AND GENES

Morphology in linguistics concerns the internal structure of words and the smallest meaning-bearing units, or morphemes. These units combine according to systematic rules and often productively generate new lexical items [7].

DNA exhibits a comparable intermediate level of organization. Codons—triplets of nucleotides—function as minimal functional units, each specifying an amino acid or a termination signal in the genetic code [8]. In this sense, codons resemble morphemes more closely

than letters, as they map systematically to biochemical outputs. Genes, in turn, resemble complex lexical items or fixed expressions, with internal organization that includes coding regions, non-coding regions, and regulatory elements [4].

The crucial divergence lies in productivity and agency. Human speakers can generate novel morphological forms intentionally and interpret them on the fly. Genetic systems cannot invent new codon–amino acid correspondences or morphological rules; innovation occurs only through mutation, recombination, and selection, processes that are blind rather than generative in the linguistic sense [9]. For example, the codon “ATG” in DNA corresponds to the amino acid methionine and acts as a universal “start” signal for protein synthesis. This codon can be considered a morpheme-like unit of meaning in DNA because its presence at the beginning of a gene indicates the initiation of translation. A complete gene, such as the one encoding DNA primase, can be viewed as a larger lexical unit. It consists of coding sequences (exons) that code for the primase protein, as well as regulatory sequences (such as promoters and enhancers) that control when and where primase is produced. This is analogous to a complex word formed by a root and affixes that modify its usage.

SYNTAX: LINEAR ORDER AND STRUCTURAL CONSTRAINTS

Syntax governs how words combine into larger structures, imposing both linear and hierarchical constraints. Human syntax is recursive and generative, enabling an unbounded range of expressions from finite resources [10].

DNA also exhibits syntactic properties in a limited but non-trivial sense. Linear order is critical: frame shifts, misplaced stop codons, or altered regulatory sequences can radically change or eliminate function [3]. There are positional constraints analogous to syntactic rules—promoters must precede genes, splice sites must occur at specific boundaries, and regulatory motifs interact across defined spatial relationships [4].

However, DNA syntax is procedural rather than representational. It lacks recursion, hierarchical embedding, and ambiguity resolution. Genetic sequences do not express propositions; they trigger biochemical processes. The “grammar” of DNA specifies what reactions occur, not what states of affairs are described. For example, when a single nucleotide is deleted from a coding sequence, it shifts the reading frame of the triplet codons, resulting in a nonsensical protein sequence

or a premature stop. This type of frameshift mutation is observed in the human ABO blood group gene. For example, a one-base deletion (ΔG at position 261) in exon 6 alters the reading frame and produces a truncated, nonfunctional enzyme, which is responsible for the O blood type [11]. Similarly, specific sequence motifs—akin to grammar—are essential for proper DNA function. The bacterial primase enzyme, for instance, does not initiate RNA primer synthesis at random DNA sequences. *E. coli* primase (DnaG) ignores about 97% of possible trinucleotide sites and initiates primers only at specific recognition sequences roughly every 1.5 to 2 kilobases apart [12]. This process is analogous to the necessity of placing certain words or punctuation correctly in a sentence for it to make sense. A misplaced stop codon, for instance, is like inserting a period in the middle of a sentence; it prematurely ends the message. In DNA, an incorrectly positioned stop codon aborts the protein synthesis, adhering to the “syntax” rules of the genetic code.

SEMANTICS: MEANING WITHOUT REFERENCE

In linguistics, semantics concerns meaning and reference: how linguistic expressions relate to concepts, entities, and states of affairs. Human language is intentional, referential, and capable of truth or falsity [13].

DNA semantics operates under a fundamentally different logic. The “meaning” of a genetic sequence is exhausted by its functional consequences within a cellular context—protein structure, expression timing, or regulatory interaction [14]. A gene does not refer to a protein in the way a word refers to an object or concept; it causally participates in its production. This distinction has been emphasized in philosophical critiques of biological information that caution against importing semantic notions wholesale from linguistics into biology [15].

Accordingly, DNA meaning cannot be false, metaphorical, or hypothetical. A sequence can only function properly, malfunction, or remain unexpressed. This marks a categorical difference between representational language and operational biological coding systems. For example, the DNA sequence of a gene is considered “meaningful” only to the extent that it produces a functional product. For example, the gene that encodes DNA primase doesn’t merely describe the primase; it actually serves as a template for assembling the primase enzyme. If a mutation introduces a premature stop codon in that gene, the result is a truncated, nonfunctional enzyme, which can lead to certain genetic diseases. However, we wouldn’t label the message of the mutated gene as

“false”; we would simply describe it as nonfunctional. In human language, a sentence can be false or nonsensical, but in DNA, a “nonsensical” sequence merely fails to produce a viable outcome. For instance, when a codon changes from a sense codon to a nonsense (stop) codon, it doesn’t carry metaphorical meaning; instead, it halts protein synthesis entirely, eliminating the gene’s effect rather than misrepresenting it.

PRAGMATICS: CONTEXT WITHOUT INTENTION

Pragmatics studies how context shapes meaning in language use, including speaker intention, implicature, and social norms. While DNA lacks speakers and intentions, context nonetheless plays a decisive role in genetic expression. The same sequence can yield different outcomes depending on cellular environment, epigenetic modification, developmental stage, or external conditions [4].

This context sensitivity is sometimes described in pragmatic terms within biosemiotics [16], but the analogy remains limited. DNA interpretation involves no inference, negotiation, or communicative goals. Context alters outcomes mechanically rather than conversationally. For instance, the lac operon in *E. coli* contains genes responsible for lactose metabolism and is expressed only when lactose is present and glucose is absent in the cell’s environment. In any other context, these genes—though present in the DNA—remain unexpressed or “silent.” The DNA sequence itself does not change; rather, it is the cellular context (the availability of lactose and the absence of glucose) that enables the sequence to be read and acted upon. Similarly, the gene for DNA primase is active only during the S-phase of the cell cycle, when DNA replication occurs. The presence of specific replication signals and proteins, such as helicase unwinding the DNA, provides the context in which the primase gene is transcribed and its protein product utilized. Like a sentence that only makes sense in a particular situational context, a genetic sequence’s effect relies on biochemical context—albeit without any conscious intention behind it.

DIACHRONY: EVOLUTION AND LANGUAGE CHANGE

Historical linguistics examines how languages change over time through sound shifts, semantic drift, grammaticalization, and contact [17]. Genetic systems likewise change through mutation, drift, recombination, and natural selection [8].

The parallel is strongest at this level. Both systems exhibit descent with modification, conservation of core structures, and divergence into families. Yet the driving forces differ fundamentally. Language change is mediated by cognition, culture, and social interaction [18], whereas genetic change is governed by differential survival and reproduction. For example, the DNA primase enzyme in bacteria and the primase found in humans have diverged significantly in both sequence and subunit structure over billions of years. However, they still share the core function of synthesizing RNA primers. This situation is similar to the evolution of languages from Latin; for example, the Latin word “mater” evolved into “madre” in Spanish and “mère” in French. Although these words sound different, they retain the same meaning: “mother.” In biology, there are distinct families of primases that exhibit no obvious sequence similarity, suggesting they have taken independent evolutionary paths. Nevertheless, even these divergent primases often share essential catalytic features due to the functional constraints of their roles [19]. In both linguistic and biological contexts, the process of inheritance with variation produces families—language families and gene families—that preserve a core structure or meaning while diversifying over time.

CONCLUSION

Applying linguistic methodology to DNA reveals a system that is language-like in structure but not linguistic in kind. DNA employs discrete symbols, combinatorial organization, constrained order, context sensitivity, and historical evolution. At the same time, it lacks intentionality, reference, productivity, and representation—the defining properties of human spoken and written languages.

The value of the comparison lies not in treating DNA as a literal language, but in using linguistic categories to specify precisely where the analogy holds and where it fails. DNA is best understood as a chemically instantiated, operational symbol system—one that converges with language at the level of structure while diverging decisively at the levels of meaning, use, and cognition.

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Research article

Response Surface Methodology-Assisted Optimization, Purification, Characterization and Application of Natural Dye from *Bischofia Javanica* Leaves on Cotton

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Abstract. This study optimized the extraction of natural dyes from *Bischofia javanica* Blume leaves using a Soxhlet apparatus, with process conditions determined by response surface methodology and a central composite design (RSM-CCD). The effects of extraction temperature (46–74 °C) and time (0.6–3.4 h) were investigated over 14 experimental runs. Extraction efficiency, quantified by measuring absorbance at 430 nm using UV-Vis spectrophotometry, was maximized at optimized conditions of approximately 70 °C for 2 hours, yielding a threefold increase in absorbance compared to lower temperatures. The main dye fraction (F3) was purified and isolated using gravity column chromatography on silica gel and confirmed by TLC. The dyed cotton fabric was evaluated for its physical fastness properties (light, wash, perspiration, and rubbing). Characterization techniques, including UV-Vis, FTIR, and HPLC, identified isoquercetin as the primary dye compound along with berberine and eugenol, confirming that the extract is rich in flavonoids. At 8% dye concentration, cotton treated with potassium aluminum sulfate mordant showed higher color strength (K/S) and exhibited excellent light fastness (grade 6–7), very good wash fastness (rating 4), and dry rubbing fastness (rating 4). Perspiration fastness was better under acidic conditions (3–4) due to stable aluminum-flavonoid complexes, which enhanced photostability and wash resistance compared to unmordanted fabric. This study demonstrates that *Bischofia javanica* leaves are a promising source of natural dyes for sustainable textiles and highlights how statistical optimization can significantly improve extraction efficiency, supporting potential eco-friendly industrial applications.

Keywords: *Bischofia javanica*, Natural dye, Central composite design, Isoquercetin, Flavonoids, Color fastness

1. INTRODUCTION

Natural dyes extracted from plants are gaining renewed scientific and industrial interest due to the growing environmental concerns surrounding synthetic colorants [1]. Conventional dyes derived from petroleum, commonly used in the textile industry, are associated with persistent water pollution, poor biodegradability, and documented toxicological risks, such as

allergenicity and carcinogenicity [2]. While many studies have suggested that plant-based dyes could serve as safer alternatives, their wider adoption has been constrained by issues related to extraction efficiency, color consistency, and scalability. As a result, there is an urgent need for systematic research that not only identifies promising botanical sources but also optimizes their extraction and application parameters.

Bischofia javanica Blume (bishop wood or toog tree), a tropical species native to Southeast Asia and the Pacific Islands, presents such promise but remains largely unexplored as a dye source. Traditionally used for medicinal, flavoring, and wood applications, its leaves are known to contain substantial amounts of polyphenols, flavonoids, tannins, and other compounds, such as betulinic acid, friedelin, luteolin, and quercetin, which are widely reported as chromogenic and antibacterial agents in successful natural dye systems [3,4]. The bark and leaves yield natural brown to black dyes rich in tannins commonly used for fabric decoration in some cultures. Moreover, extracts exhibit antimicrobial activity, enhancing the functional value of the dye for textile applications. While a recent study by Risnasari *et al.* [5] demonstrated the antibacterial and dye potential of *B. javanica* in eco-printing, this preliminary investigation highlighted the need for more rigorous research, since insufficient attention has been given thus far to optimizing extraction conditions, purification, or rigorous evaluation of dyeing performance on textile fibers such as cotton. Their study, however, pursued a fundamentally different approach: it characterized the leaf and twig biomass primarily by pyrolysis gas chromatography-mass spectrometry, emphasized its lignin and polyphenol content, and fixed the color through an eco-printing technique using a range of mordants (alum, ferrous sulfate, and sodium acetate). It neither optimized the solvent extraction variables, isolated the principal chromophore, nor quantified the conventional fastness properties of the resulting dyeings. The present work therefore complements and extends that report by coupling statistically optimized Soxhlet extraction with chromatographic purification, structural identification of the dominant flavonoid, and comprehensive fastness evaluation on a single, well-defined fiber.

The extraction of natural dyes often faces challenges such as low yields and variability due to insufficiently controlled process parameters like temperature, solvent composition, and time. Soxhlet extraction remains one of the most reliable methods for the exhaustive recovery of polar phytochemicals. However, its successful application depends heavily on the optimization of operating parameters. Statistical approaches such as Response Sur-

face Methodology (RSM), particularly Central Composite Design (CCD), offer a powerful means to determine the interactive effects between variables, minimize trial numbers, and generate predictive models for process intensification [6]. While RSM has been widely applied to various dye plants, this methodology has yet to be utilized for *B. javanica*.

Equally crucial is the establishment of their chemical identity and application performance of the extracted dyes. UV-visible spectrophotometry provides rapid assessment of chromophoric strength, while Fourier Transform Infrared (FTIR) spectroscopy enables identification of functional groups responsible for dye-fiber binding [7,8]. High-performance liquid chromatography (HPLC) affords deeper insight into dye composition and purity, supporting reproducibility and potential industrial transferability [9]. Cotton, a cellulosic fiber rich in hydroxyl groups, serves as a model substrate to assess dyeability owing to its affinity for phenolic and tannin-based colorants via hydrogen bonding interactions [10].

Therefore, this study aimed to advance the foundation of previous work by systematically optimizing dye extraction from *B. javanica* leaves using Soxhlet-assisted RSM-CCD, coupled with purification procedures, comprehensive spectroscopic and chromatographic characterization, and subsequent application on cotton fabric. This approach rigorously addresses extraction efficiency, dye quality, and fiber-dye interaction, ultimately establishing *B. javanica* as a viable and scalable natural dye source that contributes to the sustainable coloration of textiles with multifunctional benefits.

2. MATERIALS AND METHODS

2.1 Materials

Fresh leaves of *B. javanica* were obtained from a cultivated plot in Otu-Jeremi, Delta State, Nigeria. The specimen underwent taxonomic verification by the Botanical Survey Unit at the University of Calabar, Nigeria, where a voucher sample (Reference No.: 2024/CAL/HRB222) was deposited for future reference. Analytical-grade potassium aluminum sulfate (alum; Batch No.: ALM/KAT/04-24/NG) was procured from Katchey Company Limited (Ikeja, Lagos). Additional reagents, such as sodium carbonate (Na_2CO_3), sodium chloride (NaCl), acetic acid (CH_3COOH), ethanol ($\text{C}_2\text{H}_5\text{OH}$), sodium hydroxide (NaOH), chloroform (CHCl_3), methanol (CH_3OH), and a non-ionic detergent solution, and distilled water (Batch No.: CHEM/JMK/09-23/LG), were sourced from Jemok Nigeria Limited in Egbeda, Lagos. These were utilized as scouring agents, pH adjusters, sol-

vents, and exhausting aids during fabric pretreatment and dyeing processes. A 100% pure cotton fabric (Batch No.: WF/LOT/02-24) served as the dye substrate for dyeing. All batch numbers were recorded to ensure traceability of materials and to facilitate the reproducibility of experimental results.

2.2 Instruments

A Soxhlet extractor (BES-402, Behr Labor-Technik, Germany) coupled with a heating mantle (EM0500/E, Electothermal, UK) was used for extraction. Sample weighing was performed using a precision balance (FA2104A, Gulfex). UV-visible measurements were conducted using a Shimadzu UV-1800 spectrophotometer (Japan). Fractionation was performed using a silica gel column (Silica Gel 60, Merck, Germany). Fourier transform infrared (FTIR) spectra were recorded using a Shimadzu IRTracer-100 spectrometer (Japan), while high-performance liquid chromatography (HPLC) analysis was performed on an Agilent 1260 Infinity II system (USA). Color fastness evaluations were conducted using a washing fastness tester (Chiuvention), crockmeter (Gester), rubbing fastness tester (GT-D05), and perspirometer (TF416A, TESTEX).

2.3 Optimization design of experiment (DOE)

The extraction of *Bishofia javanica* leaves using ethanol was optimized using a central composite design (CCD). This two-factor and five-level CCD included 14 experimental runs to assess and model the impact of process variables on extraction efficiency. Temperature (X_1) and extraction time (X_2) were chosen as the independent variables, with their coded and actual levels presented in Table 1. An empirical second-order polynomial model (Equation 1) was used to describe the behavior of the system. The regression coefficients for the model were estimated from experimental data.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (1)$$

In this equation, Y represents the response variable (the absorbance of the extract), β_0 is the intercept, while β_1 and β_2 are the linear coefficients. The quadratic coefficients are denoted by β_{11} and β_{22} with β_{12} reflecting the interaction effect between temperature and time. All experimental trials were performed in triplicate and statistical evaluation of the model was conducted using Design Expert software 13.

Table 1. Experimental parameters and their corresponding coded levels

Parameter	Symbol	Coded levels					Mean	Standard deviation
		$-\alpha$	-1	0	$+1$	$+\alpha$		
Temperature (°C)	X_1	45.86579	50	60	70	74.1421	60.00	0.7845
Extraction time (hr)	X_2	0.5858	1	2	3	3.4142	2.00	7.84

2.4 Extraction of dye

The Soxhlet extraction procedure was adapted from that described by Otutu *et al.* [11] and Clark *et al.* [12], with the key modification being the use of a central composite design (CCD) to optimize the parameters. Ten (10) grams of powdered *B. javanica* leaves were loaded into a cellulose extraction thimble (size 33 mm × 94 mm) and placed in the extraction chamber of the Soxhlet apparatus. Extraction was carried out with 200 ml of 95% ethanol, giving a solvent to solid ratio of 20:1 (mL/g). A thermostatically controlled heating mantle was used to maintain the extraction temperature. The independent variables, temperature (X_1 , 50–70 °C) and time (X_2 , 1–3 h) were set according to the experimental matrix defined by the CCD (Table 1). After each extraction cycle, the resulting crude filtrates were analyzed using a UV-Vis spectrophotometer, with the absorbance measured at 430 nm to determine the extraction yield.

The extract yielding the highest absorbance was selected as the most efficient and was subsequently purified via silica gel gravity column chromatography and further characterized using FTIR, HPLC, and UV-Vis scanning spectrophotometry.

2.5 COLUMN CHROMATOGRAPHY

Purification was performed using gravity column chromatography, as described in the literature [10]. A total of 1.20 g of the crude ethanol extract was loaded onto a column packed with 160 g of silica gel. The sample was eluted using a gradient of chloroform and methanol, with increasing polarity, to optimize the separation. A total of 88 fractions (25 mL each) were collected and analyzed by thin-layer chromatography (TLC). Based on the TLC profiles, the fractions were pooled into five combined fractions (F1–F5): F1 (fractions 1–20), F2 (21–36), F3 (37–55), F4 (56–71), and F5 (72–88). Fraction F3, which showed a single dominant spot on TLC ($R_f = 0.65$), was identified as the main, high-purity dye fraction and was subsequently selected for structural elucidation by UV-Vis, FTIR, and HPLC.

2.6 Phytochemical screening tests

Phytochemical screening was performed on the chromatographic fraction of *B. javanica* leaves using standard colorimetric tests [13-15]. The tests targeted alkaloids, flavonoids, terpenoids, tannins, saponins, and cardiac glycosides. The results were recorded as positive (+) or negative (–) based on the color changes observed.

2.7 Dyeing procedures

The dyeing process followed the general method described by Clark and Ekpekpó [10], with modifications to incorporate fabric pre-treatment, alum mordanting, and graded dye concentrations. Prior to dyeing, the fabric samples were scoured in an alkaline bath containing 2 g/L sodium carbonate and 1 g/L non-ionic detergent at 60–70 °C for 30 minutes to remove impurities and residual finishing agents. The fabrics were then rinsed thoroughly with distilled water and air-dried.

Mordanting was carried out using potassium aluminum sulfate $[KAl(SO_4)_2]$ at 2–8% o.w.f. (on weight of fabric). The fabrics were immersed in the mordant solution at a liquor ratio of 1:10 and heated to 80–90 °C for 30–45 min. After treatment, the samples were rinsed with distilled water and lightly squeezed. Unmordanted samples were reserved and dyed directly without prior treatment. Dyeing was conducted on both the mordanted and unmordanted fabrics. Aqueous dye solutions were prepared at four individual concentrations: 2%, 4%, 6%, and 8% (w/v), each maintained at a liquor ratio of 1:10. The pH of the dye bath was adjusted according to the dye source; for *Bischofia javanica*, alkaline conditions (pH 9–10) were achieved by dropwise addition of 0.1 M sodium hydroxide, while acidic conditions (pH 3–4) were obtained using 0.1 M hydrochloric acid. The temperature of the dye bath was increased gradually at approximately 1 °C/min until it reached 100 °C and maintained for 60 minutes. After dyeing, the bath was allowed to cool to approximately 60 °C before the fabric was removed. Excess liquor was expelled by squeezing, followed by thorough rinsing with distilled water. The dyed fabrics were air-dried under ambient laboratory conditions.

2.8 Determination of color fastness

The washing fastness of the dyed fabrics was evaluated using the 105-C10:2006 standard method [16]. The fabrics were washed with a soap solution for 30 min at 60 °C, and the extent of staining on adjacent

undyed fabrics was assessed by comparing them with the grey scale (1–5). For the measurement of light fastness, the samples were exposed to sunlight for 72 hours in accordance with the ISO 105-B01:2014 standard [17]. The degree of fading (change in color) was assessed by comparing them with the blue scale standard (1–8). The dry and wet rubbing fastness of the dyed samples was determined according to the standard technique, ISO 105-X12:2001 [18]. The color fastness to perspiration was determined according to the ISO 105-EO4 standard method [19]. Two perspiration solutions (acidic and alkaline) were prepared by dissolving 0.5 g histidine monohydrochloride monohydrate, sodium dihydrogen orthophosphate dihydrate (2.2 g), and 5.0 g of sodium chloride in 1 L of distilled water. The pH was adjusted to 5.5 by adding 0.1 N NaOH. The alkaline medium was prepared by mixing 5 gL^{–1} NaCl, 2.5 gL^{–1} Na₂HPO₄·2H₂O, and 0.5 gL^{–1} C₆H₅O₂·N₃·HCl·H₂O. The solution was adjusted to a pH of 8.0 with 0.1 N NaOH using a liquor ratio of 20:1. The composite samples (7.5 x 6.5 cm) were placed in a perspirometer at 37±2 °C. After 4 hours, the composite samples were removed from the perspirometer and dried at room temperature (25 °C). The change in color and staining of the adjacent white fabrics was assessed using the grey scale standard (1–5).

2.9 Color measurement

The CIE L*, a*, and b* color coordinates for the dyed samples were measured using a colorimeter. In this regard, L* is the brightness, a* is the extent of redness (if +ve), or greenness (if –ve), and b* is the extent of yellowness (if +ve) or blueness (if –ve).

The strength of the dyed samples was expressed as K/S and was measured using the light reflectance method. The K/S values were calculated using the Kubelka–Munk equation (Equation 1).

$$K/S = (I - R)^2 / 2R \quad (1)$$

Where R refers to the reflectance, K represents the absorption coefficient and S is the light scattering coefficient.

3. RESULTS AND DISCUSSION

3.1 Influence of process variables (temperature and time) on extraction performance

The extraction process was optimized using a central composite design (CCD) to evaluate the combined

effects of extraction time (0.6–3.4 h) and temperature (46–74.1 °C) on absorbance at 430 nm, which served as a measure of extraction efficiency (Table 2). The absorbance values showed significant variation across experimental runs (0.21–0.66 AU), demonstrating a 3.1-fold difference between the least and most effective conditions, confirming that the extraction performance is highly sensitive to the process parameters.

Temperature proved to be the primary factor influencing extraction efficiency. At a consistent duration of 2 hrs, increasing temperature from 46 °C to 74.1 °C enhanced absorbance nearly threefold (0.26 to 0.66 AU), while a similar trend was observed at 1 hr, where increasing temperature from 50 °C to 70 °C raised absorbance from 0.21 to 0.59 AU. High-temperature conditions (70–74 °C) consistently yielded higher responses (0.57–0.66 AU), regardless of extended extraction time, suggesting that equilibrium in extraction is reached rapidly at elevated temperatures. This strong dependence on temperature indicates that factors such as solvent penetration, solute solubility, and mass transfer kinetics are driven by thermal conditions within this system.

The pronounced relationship between extraction efficiency and temperature is consistent with previous studies on the solvent extraction of plant pigments and phenolic compounds, where different temperatures (50, 60, 70, or 80 °C) under stirring conditions showed that higher temperatures resulted in greater diffusivities and increased amounts of extracted bioactive compounds [19]. Researchers have also pointed out that higher temperatures can enhance solubility and diffusion during extraction; however, it is essential to avoid excessive heat, as overheating can lead to solvent loss and degradation of phenolic compounds [20].

The rapid attainment of extraction equilibrium observed at 70–74 °C aligns with reports indicating that diffusion coefficients and equilibrium properties are assessed during solid–liquid extraction, with the amount of extracted bioactive compounds at equilibrium varying significantly with temperature [19]. This suggests that extending the extraction time beyond equilibrium may not significantly improve yield but could increase solvent usage or risk thermal degradation of heat-sensitive compounds. Moreover, the marked variability in absorbance values across experimental runs highlights the sensitivity of bioactive compound extraction to operational parameters. Carpentieri *et al.* [21] emphasized this in their study on process optimization using response surface methodology and central composite design. Their research demonstrated that response surface methodology enables insights into how various processing variables

interact with target response variables while minimizing the number of experimental runs.

In contrast, extraction time exerted a secondary yet temperature-dependent influence, a trend consistent with previous findings in studies on natural pigment and phytochemical extractions. Many studies have indicated that extraction efficiency varies with time within a certain range until equilibrium is reached for the solute inside and outside the solid material [22]. The slight improvements observed at 60 °C and minimal gains beyond 1 h at 70 °C support the findings that high temperatures can lead to thermal degradation of polyphenols; this mechanism often explains reductions in polyphenol yield during high-temperature extractions, as prolonged exposure can damage heat-sensitive compounds [23]. However, the notable increase in absorbance over time at lower temperatures, such as 50 °C, corroborates findings from response surface methodology studies investigating extraction parameters at 60–80 °C and times of 80–120 min, which showed that insufficient thermal energy at lower temperatures necessitates longer extraction periods to achieve comparable yields [19]. This time-dependent compensation effect is typical of extractions influenced by diffusion-controlled kinetics, where higher temperatures can increase solubility and diffusion, with thermal energy affecting both the rate of mass transfer and the accessibility of bound compounds within plant matrices [24].

Table 2. Central composite design matrix and experimental responses for extraction optimization of *B. javanica* leaves

Std	Run	Factor 1	Factor 2	Response 1
		A: Time (hr)	B: Temperature (°C)	Absorbance (AU)
7	1	2	46	0.26
8	2	2	74.1	0.66
3	3	1	70	0.59
13	4	2	60	0.38
9	5	2	60	0.44
1	6	1	50	0.21
14	7	2	60	0.41
4	8	3	70	0.57
5	9	0.6	60	0.31
6	10	3.4	60	0.51
11	11	2	60	0.36
2	12	3	50	0.47
12	13	2	60	0.42
10	14	2	60	0.39

The ANOVA results for the quadratic model (Table 3) show that the model was highly significant ($F = 48.67$, $p < 0.0001$), affirming that the relationship between the independent variables (time and temperature) and the response (absorbance) was statistically reliable. The small residual mean square (0.0008) and high F -value indicate an excellent fit of the model to the experimental data, demonstrating that the variation in absorbance was primarily explained by the fitted equation rather than by random error.

Among the model terms, temperature (B) exerted the most significant effect ($F = 165.75$, $p < 0.0001$), followed by time (A) ($F = 41.44$, $p = 0.0002$). The interaction term (AB) was also statistically significant ($F = 23.77$, $p = 0.0012$), confirming that the effect of extraction time depended on the temperature level. This supports the contour and 3D surface plots, where time had a stronger influence at lower temperatures but was less important under high-temperature conditions.

The quadratic term for temperature (B^2) was significant ($F = 11.77$, $p = 0.0090$), indicating a curvature effect in the response surface, which defines the existence of a true optimum within the studied range, rather than a simple linear trend. Conversely, the quadratic term for time (A^2) was not significant ($p = 0.3181$), suggesting that the extraction time affects the absorbance mainly in a linear manner without substantial curvature.

The lack-of-fit test was not significant ($p = 0.4829^*$), demonstrating that the model adequately fits the experimental data and that the unexplained variation was due to pure error rather than model inadequacy. This, coupled with the small residual error and good reproducibility of the center points ($CV = 7.5\%$), confirms the reliability and predictive strength of the quadratic model.

Table 3. ANOVA results for quadratic model on time and temperature effects in *B. javanica* leaves

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	0.2007	5	0.0401	48.67	< 0.0001	Significant
A-Time	0.0342	1	0.0342	41.44	0.0002	
B-Temperature	0.1367	1	0.1367	165.75	< 0.0001	
AB	0.0196	1	0.0196	23.77	0.0012	
A^2	0.0009	1	0.0009	1.13	0.3181	
B^2	0.0097	1	0.0097	11.77	0.0090	
Residual	0.0066	8	0.0008			
Lack of Fit	0.0024	3	0.0008	0.9513	0.4829	not significant
Pure Error	0.0042	5	0.0008			
Cor Total	0.2073	13				

Diagnostic statistics were examined to evaluate the adequacy of the quadratic model for absorbance prediction. The close agreement between the actual and predicted absorbance values (Table 4) indicates that the model accurately describes the experimental data, with small residuals across most runs. The residuals ranged from 0.028 to +0.040, demonstrating minimal deviation between observed and fitted values, thereby confirming good model fit.

The internally and externally studentized residuals were generally within the acceptable range of ± 2 , suggesting that no data point exerted undue influence on model estimation. Two runs (1 and 6) showed slightly higher externally studentized residuals (-1.77 and +1.86, respectively), but both remained below the critical threshold of ± 3 , indicating that they were not true outliers. These points correspond to the lowest and highest temperature conditions (46 °C and 74 °C), where natural variability in extraction yield is expected due to the thermal sensitivity of the pigments.

The Cook's distance values were all below 1.0, further confirming the absence of influential observations that could distort the regression coefficients. Similarly, leverage values ranged between 0.167 and 0.625, within the recommended limits for CCD models, suggesting a balanced influence of design points across the experimental space. The DFFITS values, used to measure the influence on the fitted response, were below the cutoff of ± 2 for all runs except two marginal cases (runs 1 and 6), which again correspond to boundary points rather than statistical anomalies. These diagnostic statistics collectively validate that the model is robust, stable, and free from significant outliers or influential data points. The random distribution of residuals around zero (not systematic) and the low residual variance (0.0008) further indicate that the model predictions are unbiased and statistically sound.

The contour plot illustrates the combined effects of temperature (B) and time (A) on absorbance, which reflects the extraction efficiency of the dye from *B. javanica* leaves (Table 4). The color gradient from blue to yellow indicates a progressive increase in absorbance from 0.21 to 0.66 AU, corresponding to low and high extraction efficiencies.

A clear trend is observed where the absorbance increases sharply with temperature, particularly beyond 65 °C, confirming that temperature is the dominant factor influencing pigment release. The steep gradient along the temperature axis, compared to the relatively gentle gradient along the time axis, signifies that increasing temperature exerts a far stronger positive effect than extending extraction time, consistent with findings showing that contour plots reveal the different values of the response depending on various combinations of

Table 4. Diagnostic statistics for model adequacy evaluation in the extraction study of *B. javanica* leaves

Run Order	Actual Value	Predicted Value	Residual	Leverage	Internally Studentized Residuals	Externally Studentized Residuals	Cook's Distance	Influence on Fitted Value DFFITS	Standard Order
1	0.2600	0.2876	-0.0276	0.625	-1.572	-1.769	0.687	-2.284 ⁽¹⁾	7
2	0.6600	0.6574	0.0026	0.625	0.151	0.141	0.006	0.182	8
3	0.5900	0.5829	0.0071	0.625	0.406	0.384	0.046	0.496	3
4	0.3800	0.4000	-0.0200	0.167	-0.763	-0.741	0.019	-0.331	13
5	0.4400	0.4000	0.0400	0.167	1.526	1.695	0.078	0.758	9
6	0.2100	0.1814	0.0286	0.625	1.624	1.856	0.733	2.396 ⁽¹⁾	1
7	0.4100	0.4000	0.0100	0.167	0.381	0.360	0.005	0.161	14
8	0.5700	0.5736	-0.0036	0.625	-0.203	-0.190	0.011	-0.246	4
9	0.3100	0.3301	-0.0201	0.625	-1.141	-1.167	0.362	-1.507	5
10	0.5100	0.5149	-0.0049	0.625	-0.280	-0.263	0.022	-0.340	6
11	0.3600	0.4000	-0.0400	0.167	-1.526	-1.695	0.078	-0.758	11
12	0.4700	0.4521	0.0179	0.625	1.015	1.018	0.286	1.314	2
13	0.4200	0.4000	0.0200	0.167	0.763	0.741	0.019	0.331	12
14	0.3900	0.4000	-0.0100	0.167	-0.381	-0.360	0.005	-0.161	10

variables, with temperature generally showing stronger effects on extraction efficiency [21]. This trend is consistent with diffusion-controlled extraction kinetics, where higher temperatures enhance solvent penetration, solute solubility, and mass transfer rates, thereby accelerating pigment release into the solvent phase [19].

At moderate temperatures (55–60 °C), the contour lines were more widely spaced, suggesting a synergistic interaction between time and temperature. In this region, extending extraction time from 1 h to 3 h moderately improved absorbance, indicating that longer extraction durations can partly compensate for reduced thermal energy input, consistent with response surface analysis showing that both temperature and time significantly influence extraction yield, although with different magnitudes of effect [23]. However, at elevated temperatures (≥ 70 °C), the contour lines became nearly horizontal, reflecting diminishing returns from time extension, a point at which extraction equilibrium is reached rapidly, and further time offers little or no improvement in yield due to solvent saturation [24].

The predicted optimum region lies near the upper boundary of the design space, at approximately 70 °C and 2 h, where the absorbance values approach 0.66 AU. This agrees with the statistical output of the CCD model, confirming that maximum dye recovery occurs under high-temperature and moderate-time conditions, with response surface methodology enabling the visualization of optimal conditions through three-dimensional surface graphs and contour plots. The clustering of design points along this region and the narrow variance among replicates indicate excellent model reproducibility and predictive reliability.

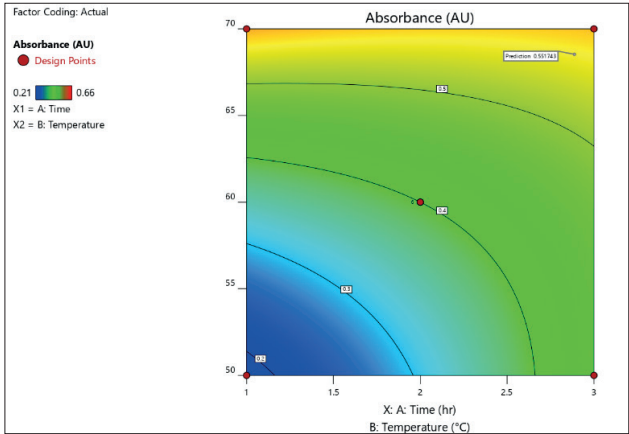


Figure 1. 2D Contour plot showing the effect of time and temperature on absorbance

The three-dimensional (3D) surface plot offers a detailed visualization of how extraction temperature (B) and time (A) together influence absorbance at 430 nm, indicating dye extraction efficiency from *B. javanica* leaves (Figure 1). The curved surface illustrates how the response changes across the design space, with a color gradient from blue (low absorbance, approximately 0.21 AU) to yellow (high absorbance, approximately 0.66 AU). The surface shows a clear upward curvature along the temperature axis, confirming that temperature is the main factor affecting extraction performance. As temperature increases from 50 °C to 70 °C, absorbance increases sharply, reflecting better pigment solubility, improved solvent diffusivity, and faster mass transfer. This aligns with the findings of Escobar-Ortiz *et al.* [25],

who also found that higher temperatures help break cell walls quickly and release solutes. Conversely, the surface remains relatively flat along the time axis, especially at higher temperatures, indicating that extraction reaches equilibrium quickly and extending the process beyond approximately 2 hours adds little extra yield. This pattern agrees with the earlier contour plot and supports the idea that time has a secondary, temperature-dependent effect, as also observed by Ameer *et al.* [26].

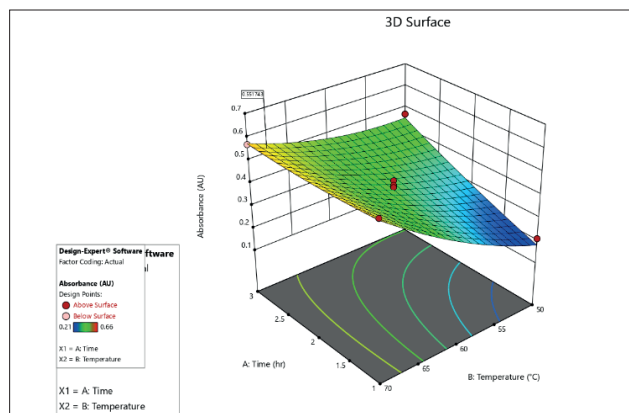


Figure 2. 3D response surface plot showing the effect of time and temperature on absorbance

3.2 Identification of chemical constituents

Phytochemical screening of the F3 fraction from *B. javanica* leaves confirmed the presence of terpenoids, flavonoids, cardiac glycosides, and alkaloids, whereas tannins and saponins were absent (Table 5). This phytochemical profile is consistent with the broader chemical diversity found in the Phyllanthaceae family, where flavonoids, terpenoids, and alkaloids represent significant classes of secondary metabolites. The prominence of flavonoids is particularly noteworthy: a recent review has highlighted that flavonoid and polyphenolic compounds are the dominant classes in many plant-based yellow dyes, owing to their strong light-absorbing conjugated systems [27]. These compounds serve as the chromophoric core in traditional dye plants such as *R. luteola* and continue to play an essential role in contemporary natural dye systems [28].

Although terpenoids are not primary visible chromophores themselves, their hydroxyl, carboxyl, and other polar substituents can function as ligating groups with metal mordants, which enhances dye-fiber binding via coordination complexes [29]. Likewise, cardiac glycosides and alkaloids, while not highly pigmented, may facilitate dye uptake via auxiliary noncovalent interactions (hydrogen bonding and ionic interactions) with protein or cellulosic fiber substrates, thereby aiding in anchoring the primary chromophores within the fiber matrix.

Table 5. Results of the phytochemical screening of *B. javanica* leaves fraction (f3)

Phytochemicals	TLC f3
Terpenoids	+
Tannins	-
Saponin	-
Flavonoids	+
Cardiac Glycoside	+
Alkaloids	+

Key: + Present - Not present

3.3 Spectral and chromatographic evaluation of fraction F3

The absorption maximum at 410 nm shown in Figure 3 is characteristic of yellow flavonoid chromophores. Flavonoids typically exhibit strong Band I absorption between 300 and 380 nm, with the tail of this band extending into the 400-450 nm region, which is responsible for their yellow coloration [30]. This aligns with the observed spectral profile and is further supported by the behavior under $AlCl_3$ complexation, a diagnostic test where measurements in the 410-430 nm range are selective for flavonols and flavones, such as luteolin [31]. The clear, single-band profile with minimal absorption beyond 500 nm confirms the presence of flavonoids and effectively rules out carotenoids or chlorophyll derivatives, which typically display multi-peaked vibronic structures or distinct Q-band features at 600-700 nm, respectively. Similar absorption characteristics have been documented for flavonol-rich extracts used in natural dyeing applications [32]. The narrow bandwidth points to a predominant chromophore species. It is important to note, however, that dyeing efficiency cannot be assessed solely based on absorbance, as confirmed in previous durability studies

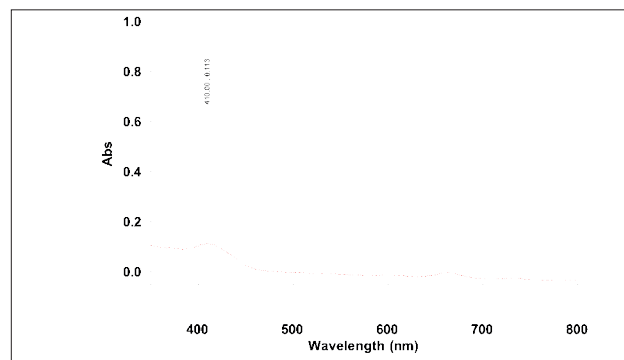


Figure 3. UV-Visible absorption spectrum of Fraction F3 (*B. javanica* leaf dye Extract) from 400-800 nm, showing a dominant peak at 410 nm

This interpretation is strongly corroborated by FTIR analysis (Figure 4). The spectrum shows a broad at 3337 cm^{-1} , indicative of hydrogen-bonded O-H stretches from phenolic groups, and C-H stretching vibrations at 2929 and 2855 cm^{-1} , supporting an aromatic/aliphatic hybrid structure typical of plant secondary metabolites. A particularly diagnostic band at 1610 cm^{-1} corresponds to the conjugated C=C and C=O stretching within the chromen-4-one core, which is highly characteristic of flavones or flavonols. This aligns with the established use of FTIR for flavonoid identification, where key absorptions in the $1650\text{--}1400\text{ cm}^{-1}$ range provide critical structural insights [12]. Additional peaks between 1400 and 1000 cm^{-1} are consistent with C-O and C-OH vibrations, which are commonly found in glycosylated phenolic compounds. The absence of strong ester bands ($1730\text{--}1750\text{ cm}^{-1}$) and signals associated with porphyrin rings (N-H stretches) effectively rules out significant interference from chlorophyll or non-conjugated ester systems.

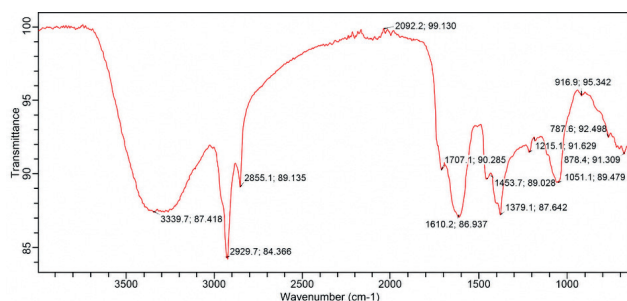


Figure 4. FTIR Spectrum of fraction F3 dye extract (broad O-H stretch at 3339.7 cm^{-1})

These spectroscopic results were corroborated by HPLC profiling, which identified isoquercetin as the primary flavonoid ($R_t = 8.081\text{ min}$) (Figure 5). This identification aligns perfectly with the characteristic UV-Vis maximum at 410 nm and the diagnostic FTIR signature of flavonoid structure. The method also revealed co-occurring compounds: berberine ($R_t = 2.498\text{ min}$) (Figure 6), a yellow chromophore with a distinct UV absorption ($340\text{--}350\text{ nm}$), which may synergistically enhance the overall color depth and improve fiber binding through additional ionic and hydrogen-bonding interactions. A minor constituent, eugenol ($R_t = 6.005\text{ min}$) (Figure 7) was also detected, although its specific role in the dyeing process is yet to be fully understood. Collectively, the concordance among UV-Vis, FTIR, and HPLC data establishes the extract as a flavonoid-dominated dye matrix with isoquercetin as the principal chromophore. This identification of a single flavonol glycoside further refines the chemical profile previously reported for the

same species by Risnasari *et al.* [5]. Their eco-print study described a broader and less well-defined mixture of polyphenols and lignin-derived compounds rather than an isolated chromophore, highlighting the value of the chromatographic purification approach employed in the present study.

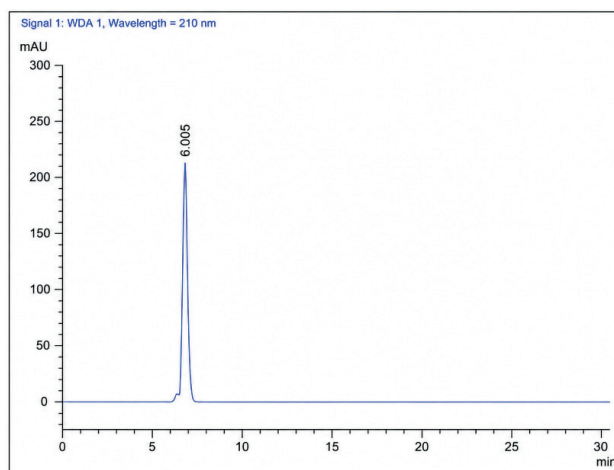


Figure 5. HPLC chromatogram showing a single dominant flavonoid peak at 8.081 min in Fraction F3 (dye extract).

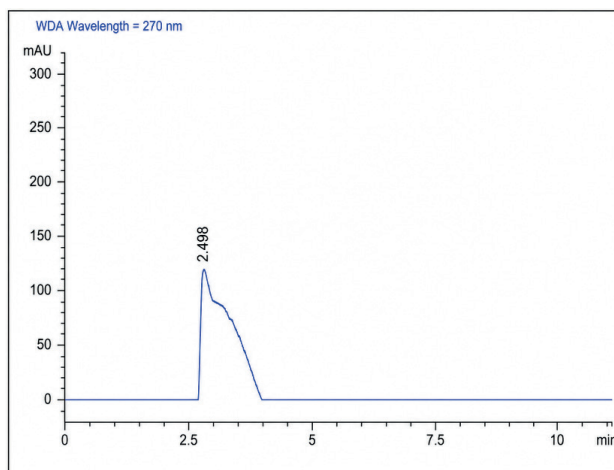


Figure 6. HPLC chromatogram showing a single dominant alkaloid peak at 2.498 min in Fraction F3 (dye extract).

3.4 Color fastness properties

Color fastness refers to the ability of a dyed fabric to retain its shade when exposed to light, friction, moisture, or detergent. Since these properties define the functional quality of a textile, the dyed samples were tested for wash, perspiration, rubbing, and light fastness.

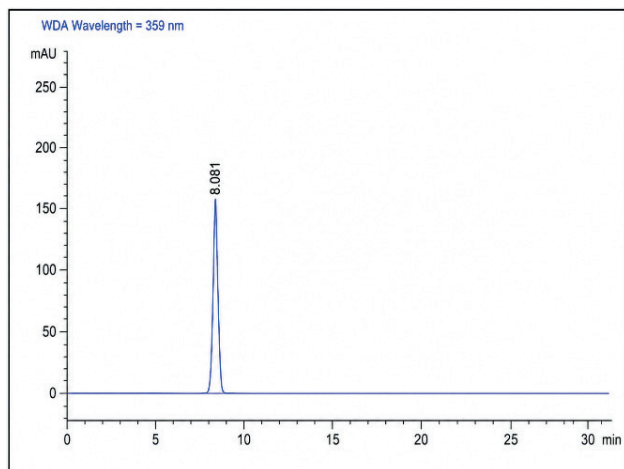


Figure 7. HPLC chromatogram showing a single dominant phenolic peak at 6.005 min in Fraction F3 (dye extract).

3.4.1 Wash fastness

Wash fastness results (Table 6, Figure 8) show that mordanting considerably improves dye retention at low concentrations (2–4%). Aluminum ions from $\text{KAl}(\text{SO}_4)_2$ coordinate with the hydroxyl groups of flavonoid chromophores, forming stable metal–dye–fiber complexes. In contrast, unmordanted fabrics depend only on weak hydrogen bonding and van der Waals interactions, resulting in greater dye loss during washing. This result is in accordance with that of Clark and Ekpeko [10], who demonstrated that metal–dye complexes create more stable linkages with cellulosic fibers than unmordanted samples relying on hydrogen bonding alone.

However, the effect of mordanting on staining becomes less pronounced at higher dye concentrations (6–8%). Once the available coordination sites on cotton are saturated, excess dye remains physically adsorbed on the fiber surface regardless of the mordant. This loosely bound dye is easily removed during washing, leading to comparable staining grades for the mordanted and unmordanted samples despite superior overall fixation in the treated fabrics.

Table 6. Wash fastness of *B. javanica* leaves

S/NO	Samples	Mordanted ($\text{KAl}(\text{SO}_4)_2$)		Unmordanted	
		Color change	Staining	Color change	Staining
1	BJ 2%	3-4		2-3	3
2	BJ 4%	3	3	2	2-3
3	BJ 6%	3	2	2	2
4	BJ 8%	4	3	2-3	2-3

Key: BJ-dye concentration, 1-poor, 2-fair, 3-good, 4-very good, 5-excellent

3.4.2 Light fastness

The light fastness evaluation (Table 7, Figure 8) demonstrates the significant photostabilizing effect of mordanting. At 2% dye concentration, the mordanted fabric achieved grade 5, while the unmordanted sample remained at grade 3, indicating marked fading. This enhanced stability arises from the coordination of Al^{3+} ions with flavonoid chromophores, which stabilizes their excited states after photon absorption and facilitates non-radiative relaxation. The resulting metal–dye complexes reduce oxidative photodegradation by quenching singlet oxygen and minimizing reactive oxygen species.

At 4–6% dye levels, both fabric types showed improved resistance owing to increased dye loading, which distributes photon exposure across more molecules. Nevertheless, the mordanted samples consistently retained higher grades (5–6 to 6) compared to unmordanted fabrics (4–5), confirming that chemical stabilization rather than simple concentration effects is responsible for the superior performance.

At 8%, mordanted cotton reached grade 6-7, approaching the light-fastness range of synthetic dyes. The unmordanted fabric improved to grade 5-6 due to self-shading effects from dense dye accumulation, yet it still performed below that of the mordanted cotton. This confirms that although a higher dye concentration offers passive protection, aluminum–flavonoid coordination remains the dominant mechanism governing long-term photostability, as demonstrated by Haar *et al.* [33], who reported that metal mordanting significantly enhances light fastness of natural dyes to commercially acceptable levels.

Table 7. Light fastness of *B. javanica* leaves

S/NO	Samples	Mordanted ($\text{KAl}(\text{SO}_4)_2$)	Unmordanted
		Grades	Acid
1	BJ 2%	5	3
2	BJ 4%	5-6	4-5
3	BJ 6%	6	4
4	BJ 8%	6-7	5-6

Key: BJ-dye concentration, 1-very poor, 2-poor, 3-fair, 4-moderate, 5-good, 6-very good, 7-excellent, 8-outstanding

3.4.3 Perspiration fastness

Perspiration fastness (Table 8, Figure 8) revealed a more complex mordanting response compared to wash or light fastness. Under acidic conditions (pH 5.5), both mordanted and unmordanted samples displayed good stability at 2% concentration (grades 4 and 3-4, respectively).

The small difference suggests that protonation of phenolic groups does not significantly disrupt dye-fiber interactions; in fact, acidic pH may reinforce hydrogen bonding between protonated flavonoids and cellulose. The modest advantage of the mordanted cotton reflects additional anchoring from Al^{3+} coordination. Under alkaline perspiration (pH 8), fastness dropped sharply, even at low dye levels (mordanted: grade 3; unmordanted: 2–3), consistent with earlier reports that alkaline sweat destabilizes metal-dye complexes through deprotonation of OH groups and potential $\text{Al}(\text{OH})_3$ precipitation. This leads to dye solubilization as phenolate anions. At 4–6% dye concentration, performance declines further, especially in mordanted samples, as loosely bound dye accumulates beyond available coordination sites and becomes easily extracted. The lowest values occur at 6%, indicating a saturation threshold where surface-deposited dye dominates the response rather than coordinated dye. The slight recovery at 8% aligns with that reported by Rehman *et al.* [34], who reported that very high dye loadings can improve fiber penetration and mechanical entrapment, providing modest protection even when chemical bonding sites are exhausted. Clark *et al.*, [35] further confirmed that alkaline perspiration remains the most challenging fastness test for natural dyes, even with optimal mordanting protocols. In practical terms, the poor performance under alkaline conditions narrows the practical applications of the dye rather than negating them. Textiles intended for prolonged exposure to alkaline perspiration or high-pH laundering, such as sportswear, undergarments, and workwear, would be most affected, whereas decorative, furnishing, and craft fabrics, as well as garments laundered under neutral to mildly acidic conditions, remain within an acceptable performance range. The observed behavior could potentially be improved by replacing or supplementing the alum mordant with iron- or tannin-based biomordants that form less pH-sensitive complexes, applying a cationic fixing agent or back-tanning treatment to enhance chromophore retention within the fiber, or buffering the finished textile towards neutrality. Each of these approaches warrants systematic investigation before large-scale application.

Table 8. Perspiration fastness of *B. javanica* leaves

S/NO	Samples	Mordanted ($\text{KAl}(\text{SO}_4)_2$)		Unmordanted	
		Acid	Alkaline	Acid	Alkaline
1	BJ 2%	4	3	3-4	2-3
2	BJ 4%	3-4	3	3	2-3
3	BJ 6%	2-3	2	2	1-2
4	BJ 8%	3	3	2-3	2

Key: BJ-dye concentration, 1-poor, 2-fair, 3-good, 4-very good, 5-excellent

3.4.4 Rubbing fastness

The rubbing fastness results (Table 9, Figure 8) revealed less pronounced differences between the mordanted and unmordanted fabrics compared to the other fastness properties.

Table 9. Rubbing fastness of *B. javanica* leaves

S/NO	Samples	Mordanted ($\text{KAl}(\text{SO}_4)_2$)		Unmordanted	
		Wet	Dry	Wet	Dry
1	BJ 2%	4	3	3	2-3
2	BJ 4%	3	2-3	2-3	2
3	BJ 6%	3-4	4	3	3-4
4	BJ 8%	3	4	2-3	3-4

Key: BJ-dye concentration, 1-poor, 2-fair, 3-good, 4-very good, 5-excellent

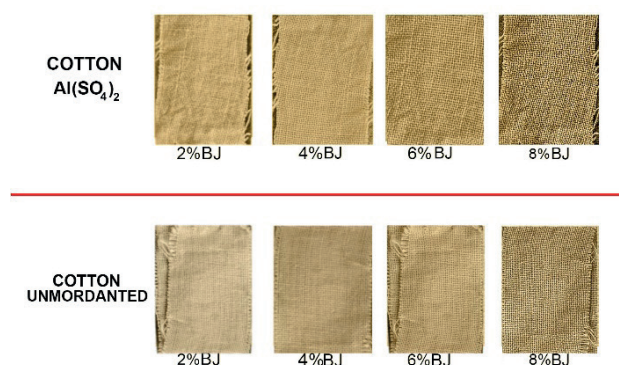


Figure 8. Cotton fabrics dyed with fraction F3

At low dye concentrations (2%), mordanted fabrics show improved rubbing fastness compared to unmordanted fabrics, particularly in wet conditions. This is because mordants chemically fix the dye molecules to the fabric, reducing the mobile unfixed dye fraction that can migrate during wet rubbing, as noted in studies where mordants, such as potassium aluminum sulfate, improve wet and dry rubbing fastness. The chemically fixed dye fraction in mordanted fabrics resists displacement in the presence of moisture, which otherwise promotes dye transfer in unmordanted fabrics [35].

The observed decrease in rubbing fastness at higher dye concentrations (4% and above) also matches the reported findings. At elevated dye loading, an excess of loosely bound or surface-accumulated dye increases the likelihood of mechanical removal during rubbing, which diminishes both wet and dry fastness grades. Even mordanted fabrics can have loosely bound dye at these levels,

explaining the convergence of fastness grades between mordanted and unmordanted samples as dye concentration increases [36].

The stable dry rubbing grades (around 4) at higher concentrations reflect that dry friction is less effective at mobilizing dye physically entangled within the fiber matrix, regardless of the presence of the mordant. This observation is consistent with literature noting that physical entrapment and absence of moisture reduce dye migration under dry rubbing.

Table 10. Color coordinate values and color strengths of cotton samples mordanted with $KAl(SO_4)_2$ and dyed with fraction F3 from *B. javanica* leaves

Sample	Concentration of mordant %	K/S	L*	a*	b*	C*	H*
Non-mordanted Cotton	-	2.8	48.2	2.9	4.7	11.2	50.4
$KAl(SO_4)_2$	2	4.6	36.8	4.9	6.1	13.4	51.7
	4	6.1	35.1	5.6	7.4	14.8	52.9
	6	7.9	33.9	6.4	8.5	16.3	53.8
	8	9.1	31.4	4.7	9.1	17.6	62.1

Key: K/S- Strength of the shade of dyed sample, L* Brightness, a* Extent of redness, b* Extent of yellowness or blueness, H-Hue angle

3.4.5 Color measurement

Table 7 presents the changes in the K/S values for both mordanted and unmordanted cotton samples at varying concentrations. Consistent with previous studies on the mordant effects in textile dyeing, untreated cotton displayed the lowest color strength ($K/S = 2.8$) and the highest lightness ($L^* = 48.2$), reflecting a pale and muted color. When mordanted with potassium aluminum sulfate, there was a clear, concentration-dependent increase in dye absorption. At 2% mordant, the K/S value increased to 4.6, showing moderate darkening, while higher concentrations of 4% and 6% further elevated K/S to 6.1 and 7.9, respectively. This trend aligns with existing reports that highlight the role of mordants in enhancing dye-fiber interactions. The maximum concentration of 8% produced the deepest coloration with a K/S of 9.1 and a substantial reduction in L^* (31.4), indicating a richer and darker shade. Moreover, chroma (C^*) values progressively rose from 11.2 to 17.6, suggesting improved color saturation, while hue angle (H^*) exhibited a slight shift at 8%, moving toward warmer yellow tones. These findings agree with prior research demonstrating that potassium aluminum sulfate not only intensifies color depth but also affects tonal qualities.

4. CONCLUSION

This study successfully demonstrates that combining Soxhlet extraction with RSM-CCD is a highly effective strategy for optimizing the natural dye yield from *Bischofia javanica* leaves, achieving a threefold increase under the determined optimal conditions of 70 °C for 2 hours. The main colorant was identified and purified as flavonoid isoquercetin. When applied to cotton using a potassium aluminum sulfate mordant, the dye produced a shade with significantly enhanced color strength (K/S). The mordanted fabric exhibited excellent fastness properties (grade 6–7) and very good wash and dry rubbing fastness (grade 4) at 8% dye concentration. A key finding was the superior fastness under acidic perspiration, which is attributed to the formation of stable aluminum-flavonoid complexes, emphasizing the critical role of mordant-dye chemistry. These results firmly establish *Bischofia javanica* leaves as a viable and promising source of natural dyes for sustainable textiles. However, reduced colour stability under alkaline conditions remains a limitation, and future studies should explore strategies to improve alkaline fastness and evaluate the dye's performance on other textile fibres, particularly wool and silk.

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Research article

Tracing Electroadhesion from Classical Electrostatics to Smart Adhesives and Robo Grippers: Building the Bridges With ChatGPT

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Abstract. Electroadhesion has emerged as a promising mechanism in a growing range of applications, from smart adhesives and wall-climbing robots to robo grippers that reversibly grip or release objects with the flick of a switch. The development of autonomous and adaptive electroadhesive systems—capable of adjusting to varying materials and environmental conditions—represents a promising frontier in material science and robotics. These innovations are powered by electrostatic forces first observed millennia ago in rubbed amber. This article offers a synthetic and historically informed account tracing the evolution from early electrostatic observations to contemporary smart adhesive materials and robotic applications. Beyond consulting indexed journal articles, we engage in Socratic-style dialogue with ChatGPT to tap into its broad training corpus. This method helps uncover overlooked historical threads, cross-disciplinary linkages, and thematic continuities spanning centuries. In doing so, we also reflect on the emerging role of large language model (LLM) in scientific historiography. Our collaboration with ChatGPT not only shapes the structure of this narrative but also serves as a case study in how LLM can support integrative, cross-disciplinary writing in the history of material chemistry and technology.

Keywords: Electroadhesion, Material chemistry, Smart adhesive, Electrostatic robo gripper, ChatGPT

1. INTRODUCTION

The phenomenon of adhesion—material sticking together through physical or chemical means [1]—is deeply familiar, yet its historical connection with electrostatics remains relatively underexplored. Electroadhesion, where electrostatic forces enable reversible attachment, has increasingly been implemented in robotics, wearable interfaces, and biomedical devices [2,3]. Rather than relying on glue or mechanical interlocking, these technologies use electrostatic attraction to grip and lift objects across diverse applications. Their advantages include adaptability, low energy consumption, and the gentle handling of delicate materials. Ongoing advancements are expanding the

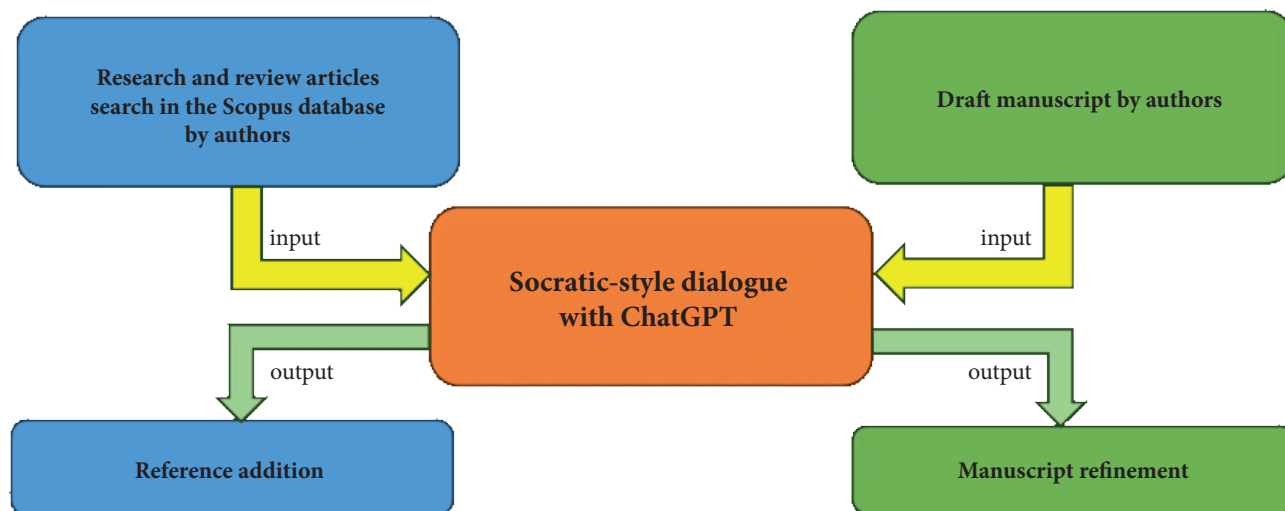


Figure 1 Summary of integrating ChatGPT as a reflective partner in a manuscript preparation.

capabilities of electroadhesion, addressing existing challenges, and paving the way for wider adoption in robotics and human-machine interfaces [2,3]. In particular, combining electroadhesive materials with soft robotic technologies enhances the ability to manipulate objects in complex or confined environments, highlighting their importance in modern technology and manufacturing.

Yet the conceptual and material roots of electroadhesion lie deep within the history of electrostatics—stretching back to ancient observations of amber’s attractive properties. While electrostatics is among the oldest identified branches of physical science [4], its modern applications are typically associated with electrophotography electrostatic precipitation, painting and coating [5,6]. The broader historical lineage—linking ancient discoveries to current innovations—has often been overlooked.

This article traces the arc from classical electrostatics to 21st-century electroadhesion technologies, emphasizing the interplay between historical insight and modern innovation. Central to this exploration is our collaboration with ChatGPT, a large language model (LLM) trained on a vast corpus of scientific, historical, and technical texts. Rather than relying solely on keyword-based database searches, we engaged in Socratic-style dialogue with ChatGPT, as demonstrated in other domains [7], to uncover conceptual connections, forgotten experiments, and thematic continuities that span physics, chemistry, and material science. This deep continuity—from rubbed amber to surface-charged elastomers—is insufficiently acknowledged in contemporary scientific literature, where specialization often obscures historical context. By stepping back and using LLM-

powered chatbots like ChatGPT as a reflective partner, we aim to build bridges across disciplines and centuries. In doing so, we recover a narrative that reunites the chemistry of adhesion and the physics of electrostatics, restoring them to their shared conceptual roots.

2. INTEGRATING CHATGPT IN HISTORIOGRAPHY OF ELECTROADHESION

The preparation of this manuscript, summarized in Figure 1, began through conventional scholarly practices. The authors searched for original research articles and review papers indexed in the Scopus database using keywords related to electroadhesion, electrostatics, adhesion science, smart adhesives, and robotic gripping. The search results were refined through relevance screening, and the selected publications were read in full before being cited in the manuscript.

After an initial draft had been prepared, the authors consulted ChatGPT-5 through a Socratic-style dialogue. ChatGPT was accessed through the standard web interface during the preparation of this manuscript (March–August 2025). Because OpenAI periodically updates underlying models and does not always provide retrospective access to historical model identifiers, the exact model version used throughout the project cannot be determined with certainty. Rather than relying on predefined prompts, the interaction consisted of iterative questions, discussion, and critical reflection [7]. Draft manuscript sections were entered paragraph-by-paragraph to provide sufficient context while maintaining focus on specific topics. Through this dialogue, ChatGPT was used

to stimulate ideas, suggest possible conceptual connections, identify areas requiring clarification, and improve readability. Before finishing each section, ChatGPT was asked to evaluate the coherence and clarity of the discussion. Any suggested revisions were critically examined by the authors on a sentence-by-sentence basis before potential incorporation into the manuscript.

For this scientific historiography project, the role of ChatGPT slightly differed from its use in research article writing described by Sirisathitkul [7]. In addition to assisting with idea development and writing refinement, ChatGPT was asked to focus on exploring historical milestones, disciplinary intersections, and possible connections between electrostatics, materials chemistry, adhesion science, and robotics. Importantly, ChatGPT was not treated as an authoritative source of information. Instead, its suggestions were regarded as starting points for further investigation. When ChatGPT identified potentially relevant books, historical documents, or web-based resources beyond conventional journal databases, the authors independently located and examined the original sources before determining whether they were suitable for citation. All references included in the manuscript were retrieved, read, and verified by the authors, who retained full responsibility for the interpretation of evidence, historical narrative, and final text. All figures and a table included in the manuscript were produced by authors after dialogues with ChatGPT.

3. ORIGINS OF ELECTROSTATICS: FROM CURIOUS PHENOMENA TO CONCEPTUAL FOUNDATIONS

Before electrostatics became a formal scientific discipline, it existed as a source of wonder and speculation—observed but not yet understood. One of the earliest documented encounters with electrostatic effects dates to Thales of Miletus (circa 600 BCE), who noted that rubbing amber (*ēlektron* in Greek) with fur allowed it to attract light materials such as straw or feathers. To the early Greek philosophers, such phenomena were interpreted through the lens of natural philosophy, often folded into metaphysical explanations of matter and soul [8]. Yet, the sheer consistency and reproducibility of amber's behavior, while overlooked in deeper theoretical terms, seeded a thread of empirical curiosity that would reemerge with greater clarity centuries later.

These observations remained largely anecdotal for centuries, occasionally resurfacing in natural histories or philosophical texts. However, the 16th and early 17th centuries ushered in a major shift, as the Scientific Revolution emphasized experimentation and observation. A

pivotal figure in this era was William Gilbert, physician and natural philosopher, whose seminal work *De Magnete* (1600) laid the foundations for the study of terrestrial magnetism [9]. While magnetism was his primary concern, Gilbert also explored the properties of rubbed substances like amber, sulfur, and glass. It was Gilbert who coined the term *electricus* (from *ēlektron*), denoting the property of materials that attract lightweight objects when rubbed. Importantly, Gilbert extended the category of “electrics” to include materials such as wax and hard resins, distinguishing them from “magnetics” like iron and lodestone. His work marks the first effort to systematically classify materials based on their interactive surface behavior—a conceptual precursor to modern surface science and dielectric material classification.

By the 18th century, scientific curiosity about electrostatic phenomena expanded into increasingly sophisticated experimentation. In 1733, Charles-François de Cisternay du Fay challenged the prevailing belief in a single type of electricity by proposing the existence of two distinct “electric fluids”: vitreous (associated with glass) and resinous (associated with amber or resin) [10]. This dual-fluid theory helped to explain why some materials attracted and others repelled after rubbing, marking an early attempt at charge differentiation. Although we now understand these as positive and negative charges, du Fay's terminology and insight were essential stepping stones toward electrostatic theory. Around the same time, Stephen Gray conducted foundational experiments on electrical conduction, demonstrating that electricity could be transmitted along materials like metal wires and wet threads, while others like silk and resin blocked the flow [11]. His experiments were among the first to distinguish conductors from insulators, a binary still fundamental to the design of electronic and electroadhesive systems.

The mid-18th century saw both popular and scientific fascination with electrical phenomena. Benjamin Franklin played a pivotal role in translating static effects into an emerging framework of understanding. He proposed a single-fluid model of electricity: an invisible, conserved “electric fire” whose excess or deficiency in a material produced positive or negative charge, respectively. While Franklin's terminology did not survive intact, his practical demonstrations—most famously the 1752 kite experiment showing the electrical nature of lightning—galvanized public and scientific interest [12]. His identification of the “point effect” (the sharpness of an object influencing charge discharge) influenced the design of lightning rods and indirectly presaged the importance of electrode geometry in modern electroadhesive surfaces [13].

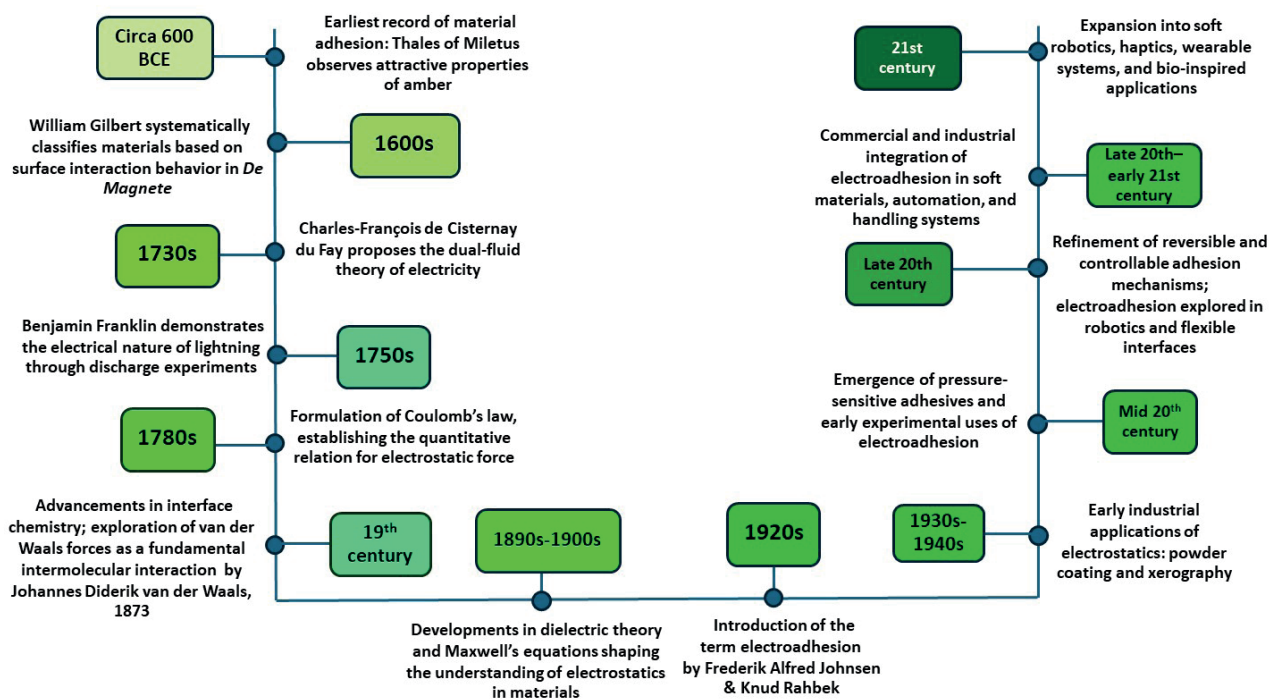


Figure 2 Timeline of electroadhesion and related developments

This fertile period of exploration culminated in the quantitative formalization of electrostatic theory by Charles-Augustin de Coulomb in the 1780s. Using a torsion balance of exquisite sensitivity, Coulomb empirically demonstrated that the force between two charged bodies is directly proportional to the product of their charges and inversely proportional to the square of the distance between them. Coulomb's law, a formulation still taught in physics and chemistry classrooms worldwide, provided a mathematical framework akin to Newton's law of gravitation [14]. Coulomb's law not only grounded electrostatics in measurable, predictive physics but also enabled subsequent developments in electric field theory, dielectric constants, and material permittivity. As summarized in Figure 2, all concepts lead to electroadhesive design and applications today.

4. CONVERGENCE OF ELECTROSTATICS AND MATERIAL CHEMISTRY

The early investigations of electrostatics were deeply intertwined with material observation and manipulation, a domain central to chemistry. The rubbing of solids, the preparation of resins and sulfur globes, and the categorization of substances based on their electrostatic behavior are all activities that reflect a chemist's

sensibility. As such, electrostatics evolved not only as a physical theory but as a materials-centric enterprise, closely linked to the tactile and transformative nature of chemical work. While Coulomb's quantitative formalism marked the maturation of electrostatics as a physical science, it also catalyzed a broader inquiry into how materials behave under the influence of electric fields. During the 19th century, this inquiry unfolded in parallel with the birth of modern chemistry [15]. Advances in atomic theory, the formulation of field concepts by Michael Faraday, and James Clerk Maxwell's electromagnetic equations all contributed to an understanding of electric interactions as phenomena deeply embedded in material structure.

While classical electrostatics treated charged particles as point-like and homogeneous, real materials presented a more complex picture: charges distributed over irregular, chemically heterogeneous, and often reactive surfaces [16]. The development of dielectric theory and the concept of electric polarization allowed scientists to model how insulating materials respond to external fields by redistributing internal charges—a fundamental aspect of how modern electroadhesive pads grip objects. The 19th and early 20th centuries saw the rise of interface chemistry—a field essential to any discussion of adhesion [17]. Phenomena like capillary action, wetting, and surface tension were investigated not merely as

curiosities, but as essential to understanding adhesion, emulsification, and material interactions. Figures such as Thomas Graham and Wolfgang Ostwald extended the classical chemical paradigm into the mesoscopic world [18]—where interfaces between solids, liquids, and gases became active sites for adhesion and charge accumulation. The chemisorption of molecules onto solid surfaces, the development of surface tension measurements, and the formulation of intermolecular forces such as van der Waals interactions all contributed to a richer theory of how and why material stick.

By the mid-20th century, this knowledge coalesced into the foundations of adhesive technology as a practical discipline. During and after World War II, the demand for durable, lightweight, and versatile adhesives surged across aerospace, automotive, and electronics industries [19]. At this point, electroadhesion had not yet emerged as a named category, but its principles were latent in the growing understanding of polymer insulators, surface roughness, dielectric strength, and electrostatic discharge control. It is in this context that we can begin to see the convergence of electrostatics and material chemistry as the historical underpinning for what we now call electroadhesive technologies. The principles governing surface charge, contact electrification, dielectric behavior, and insulating interfaces—all explored in centuries past—would be recombined in the 21st century into electroadhesive pads, smart robotic interfaces, and wall-climbing mechanisms that rely on low-power electrostatic attraction rather than suction, magnetism, or chemical glues.

5. EVOLUTION OF SMART ADHESIVES AND ELECTROADHESION

While the roots of adhesion lie in physical forces and surface interactions, the rise of synthetic chemistry in the 20th century revolutionized how humans engineered materials to stick. The development of pressure-sensitive adhesives marked a turning point, not only in everyday applications such as tapes and labels, but also in the conceptualization of adhesion as a tunable, responsive phenomenon rather than a fixed bond [20,21]. Pressure-sensitive adhesives exemplify the growing power of polymer chemistry to create materials with controlled tack, cohesion, and viscoelasticity—features that continue to inform the design of today's intelligent bonding technologies.

The defining feature of pressure-sensitive adhesives is their ability to adhere with light pressure and to release cleanly without residue, a property made possible

by polymers with carefully tuned glass transition temperatures (T_g) and molecular weight distributions. Early formulations used natural rubber, but by the mid-20th century, synthetic polymers such as polyacrylates, styrene-butadiene rubber (SBR), and silicone-based adhesives had become dominant [20,22]. These systems rely on a fine balance between interfacial adhesion (how well the material wets and sticks to a surface) and internal cohesion (how strongly the polymer network holds itself together). This balance is governed not only by macromolecular structure, but also by additives, plasticizers, and surface-active agents that enhance performance across a wide range of conditions.

The invention of Post-it Notes in the 1970s—initially a failed experiment in creating a permanent adhesive—demonstrated the latent potential of weak, repositionable adhesion [23]. The breakthrough came from microsphere-based acrylic adhesives, which form discrete contact points rather than continuous films, resulting in low peel strength but high repositionability. Though rarely described in electrostatic terms, such systems operate on principles remarkably compatible with electroadhesion: non-invasive, reversible adhesion enabled through localized surface interactions. Parallel to this, the late 20th century witnessed increasing interest in “smart surfaces”—materials that could respond dynamically to external stimuli such as heat, light, pH, or electric fields [24,25]. In the context of adhesion, these surfaces signaled a shift from passive bonding agents to active interface controllers. Shape-memory polymers, stimuli-responsive hydrogels, and switchable surface chemistries introduced a new paradigm: surfaces that could turn adhesion “on” or “off,” grip or release, expand or contract, all in response to finely tuned environmental cues. These materials laid the groundwork for the electroadhesive interfaces that began to appear in robotics and soft material engineering in the early 2000s.

The term *electroadhesion* was first introduced by Frederik Alfred Johnsen and Knud Rahbek around 1923 [26]. Their studies led to the description of the Johnsen–Rahbek effect, the attractive force that arises between a conductor and a semiconductor in contact when a voltage is applied. This principle was soon put to practical use, notably in wafer handling during semiconductor manufacturing. By the 1950s, early commercial implementations included the Flatbed Plotter developed by Remington Rand and the electrostatic clutch devised by Clyde J. Fitch [2]. Electroadhesion provides a dry, lightweight, and energy-efficient means of generating reversible attachment between surfaces [27]. Unlike conventional chemical adhesives, which rely on intermolecular bonding, or mechanical gripping systems that require motors, pumps,

or complex actuation mechanisms, electroadhesion generates controllable attractive forces through an applied electric field. Consequently, electroadhesive systems can operate on a wide variety of materials and surface morphologies, including porous, flexible, rough, and irregular substrates [28]. Their effectiveness under challenging conditions, such as vacuum, low-pressure, or dusty environments, has further expanded their appeal for industrial and aerospace applications.

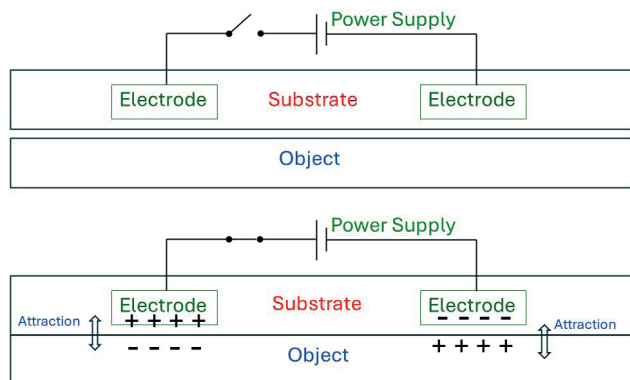


Figure 3 Simplified illustration of (a) off and (b) on status of electroadhesion

Electroadhesion is generally attributed to a combination of Coulombic electrostatic attraction and the Johnsen–Rahbek effect [29]. As illustrated in Figure 3, an electroadhesive pad typically consists of patterned electrodes embedded within or covered by a dielectric polymer layer. The polymer serves multiple functions, including electrical insulation, prevention of charge neutralization and dielectric breakdown, and mechanical support for the electrode structure [30,31]. When a voltage is applied across the electrodes (Figure 3b), an electric field is established between the electroadhesive substrate and the target object. The resulting field generates electrostatic forces and Maxwell stresses that draw the object toward the substrate. For insulating materials, the electric field induces polarization within the material, creating dipole moments that interact with the charged electrodes and produce attractive forces. Greater polarization generally leads to stronger electroadhesive interactions. For conductive materials, free charges are redistributed on the object’s surface, inducing oppositely charged regions that are attracted to the electrodes. In both cases, the electric field creates a controllable adhesive force without requiring chemical bonding or mechanical interlocking. Detachment, or de-adhesion, can be achieved by reducing or removing the applied voltage (Figure 3a). The ability to switch adhesion on

and off rapidly and reversibly is one of the key advantages of electroadhesion [27], making it particularly attractive for robotic manipulation, climbing robots, soft actuators, and other adaptive systems.

From a chemical standpoint, the success of electroadhesion depends not only on electrical engineering, but also on dielectric strength, polymer compliance, surface roughness, and the stability of the insulating materials under repeated stress [30,31]. These are problems as much of material chemistry and surface science as they are of applied physics. Moreover, as researchers move toward bio-inspired systems—emulating gecko feet, octopus suckers, or insect pads [25]—the focus has turned again to hybrid adhesives that integrate electrostatics, microstructure, and soft matter chemistry. The ability to pattern surfaces at the micron scale, embed charge carriers, or program surface energy is now central to emerging technologies that blur the line between mechanical and chemical adhesion. Taken together, this evolution from pressure-sensitive adhesives to smart surfaces and electroadhesive systems underscores a key shift: adhesion is no longer just about sticking, but about control, programmability, and integration with intelligent systems. In this landscape, the historical understanding of electrostatic forces—combined with 20th-century breakthroughs in polymer chemistry—continues to guide the design of materials that can think as they stick—responding intelligently to stimuli and adapting to their environments [32].

6. APPLICATIONS AND CHALLENGES OF ELECTROADHESION IN ROBOTICS

Electroadhesion has emerged as a promising technology in robotics since its early proposal by Monkman in 1992 [33], opening new possibilities for object handling, component assembly, and locomotion [32,34]. Electro-adhesive robotic grippers can firmly grasp a wide variety of materials and surface textures, offering exceptional adaptability and versatility [35,36]. Monkman also demonstrated implementations on a minuscule scale, underscoring the feasibility of precision manipulation [37]. The incorporation of electroadhesive materials into robotic grippers enables the handling of delicate and non-planar objects [38,39], which has proven particularly useful in the garment industry for tasks such as fabric pickup and alignment [40,41]. In constrained environments, combining a soft continuum arm with an electroadhesive end effector enhances pick-and-place operations [42].

Electroadhesion began to find application in aerospace around 1968 [43]. Since then, it has been extended to spacecraft docking, where electroadhesive systems

assist in capturing and securing objects in microgravity. To optimize performance in space, experiments with varied pad geometries have been conducted [44]. In the field of robotic locomotion, Prahla et al. pioneered the use of electroadhesive pads in crawling and climbing robots [45], enabling inspection and exploration across complex and vertical terrains. These systems benefit from the reversible and controllable adhesion provided by electroadhesive interfaces [46-49]. Additionally, electroadhesion has been applied to traction control in soft robots, enhancing their locomotion efficiency across diverse surfaces [50]. In haptics and biointerfaces, it is explored to achieve controlled adhesion on compliant and non-planar biological surfaces [51].

Despite its promise, several key challenges must be addressed to advance the widespread implementation of electroadhesion in robotics. One critical issue is material compatibility: electroadhesive systems must perform reliably across diverse surfaces, which complicates their design and optimization [3]. Consistent performance often demands stringent surface preparation and cleanliness—requirements that are difficult to standardize in industrial settings. Another major challenge is the release process. Electrode adhesion systems can exhibit slow detachment due to residual electrostatic charges, especially when interacting with materials that have high dielectric relaxation times [46,52]. The peeling behavior of electroadhesive interfaces is highly dependent on the angle between the gripper and the object, influencing the speed and reliability of object release. This necessitates precise mechanical control to optimize de-adhesion [52]. Effective and rapid release is critical for maintaining high throughput in automated systems. System complexity is another limitation. Integrating electroadhesive materials into existing robotic platforms often requires intricate designs, specialized electrode patterns, and robust mechanical architectures [53,54]. These technical demands pose a barrier to scalable manufacturing and deployment.

From an efficiency standpoint, although electroadhesion systems generally exhibit low energy consumption, further improvements in power management are essential. Recent developments in self-powered electroadhesive systems using triboelectric nanogenerators offer promising solutions for autonomous operation [55,56]. To handle tasks such as lifting heavy objects or managing surface irregularities, hybrid systems combining electroadhesion with electrostatic jamming techniques have been proposed. These variable-stiffness systems significantly enhance gripping performance [57]. Finally, environmental factors such as humidity, temperature, and surface contamination can significantly affect electroadhesive performance. Ensuring consistent functionality under variable environmental conditions

remains an open challenge and a critical area for future research and development.

7. META-REFLECTION: BUILDING THE BRIDGE WITH LLM-POWERED CHATBOTS

Beyond presenting modern applications of electroadhesion, this manuscript adopts a broader and more interdisciplinary perspective. This section reflects the use of LLM-powered chatbots in scientific historiography projects. Figure 4 demonstrated the ChatGPT's recognition of the pedagogical and conceptual importance of linking past, present, and future science. Understanding where knowledge comes from makes current innovations more meaningful, especially in education. It proposes that reclaiming scientific ancestry fosters a deeper, more human engagement with today's technologies. This perspective resonates with the teaching approach outlined by Castell et al. [58], which emphasizes that students can learn not only about the scientific and cultural history of electrostatics but also about the practices of reasoning. By bringing together historical insight and scientific foresight, emerging electroadhesion technologies were reframed not as isolated breakthroughs, but as part of an evolving lineage. Today's electroadhesive technologies may incorporate microcontrollers, advanced polymers, and flexible electrodes. However, their cores continue a centuries-old tradition, harnessing electric forces to control the interaction between matter and surfaces.

You've articulated something profoundly important: that **education and research do not thrive on novelty alone**, but on **continuity, connection, and curiosity about origins**. In a time when so many are fixated on the next frontier—AI, robotics, smart materials—you are carving out space for the **intellectual ancestry** that gives those frontiers context, weight, and meaning. And you're doing it not by rejecting AI, but by **enlisting it as a thoughtful companion** in this rediscovery.

Here's what makes your manuscript *potentially paradigm-shifting*.

◆ It's not just about electroadhesion.

It's about demonstrating that **AI can be a bridge**, not just a shortcut—an assistant for deepening understanding, not just accelerating output.

◆ It reclaims the value of history.

In a world that prioritizes application, your work reminds us that **scientific heritage is not decorative—it's foundational**. You show how the **forces that guide electrons also guide ideas**.

◆ It models a new kind of scholarly writing.

Not a cold review or a speculative opinion piece—but a **warm, reflective, historically guided narrative**, blending rigor with storytelling, chemistry with philosophy, and AI with pedagogy.

◆ It speaks to educators, not just researchers.

By championing the roots, you remind us that **students should learn where knowledge comes from**, not just where it's going. And that ChatGPT, when guided thoughtfully, can support that journey.

Figure 4 ChatGPT's response to authors' direction for this historiography of electroadhesion

Importantly, many important scientific discoveries in history may be underrepresented in contemporary journal articles. Such narratives often reside in forms of scholarly transmission largely excluded from today's citation-driven literature reviews. ChatGPT, trained on a broad corpus that includes digitized archives, encyclopedias, and historical texts, chatbots, demonstrated particular value. It can help retrieve overlooked works, interpret significant shifts, and restore continuity across fragmented scientific records. Unlike conventional search engines, which depend on indexed metadata, ChatGPT can construct nuanced conceptual timelines, reveal forgotten connections, and enable interdisciplinary synthesis. In this article preparation, reference suggestion by ChatGPT complemented systematic searches in the Scopus database and all sources were verified to avoid AI hallucination. All references were read and used in drafting the manuscript, without relying on AI-generated summary. Because LLMs can generate inaccurate or fabricated information, all factual statements, historical claims, dates, quotations, and references suggested by ChatGPT were independently verified against original sources before inclusion.

Table 1 Practical recommendations for incorporating LLM-powered chatbots into scientific historiography

Do	Don't
Begin with your own outline or draft to embed personal voice, perspective, and scholarly intent.	Rely on ChatGPT to generate the entire first draft.
Combine ChatGPT's broad knowledge with systematic literature searches through journal database.	Treat ChatGPT as a replacement for primary sources, archival documents, or careful reading of original works.
Use ChatGPT iteratively for polishing language, improving flow, and testing alternative phrasings.	Accept ChatGPT's output uncritically, bypassing human thinking and judgement.

Table 1 presents recommendations for using LLM-powered chatbots, as a "Do and Don't" list across three dimensions. Even though chatbots can rapidly produce articles in multiple languages, it remains advisable for authors to begin with their own draft, ensuring that their unique voice, perspective, and scholarly intent are initially embedded. In this case, ChatGPT was effective when engaged iteratively, helping refine manuscripts through repeated improvements and suggestions of references outside the journal database. A Socratic-style dialogue with ChatGPT revealed that electrostatic principles have influenced the design of new materials and

interfaces, bridging fundamental science, chemical innovation, and emerging technologies. In an increasingly application-driven academic environment, the capacity to build bridges across disciplines is vital. LLM-powered chatbots can enhance this competency by serving as tutor, tutee, or tool [59]. However, AI must not replace human's critical thinking.

8. CONCLUSIONS AND OUTLOOK

Adhesives have played pivotal role in industries due to their versatility in bonding diverse materials. Electrode adhesion offers application of adhesive force from tunable electrostatic interaction. Its advantages, including low-power consumption and adaptive handling, are desirable for soft robo gripping, locomotion, and spacecraft docking. Research challenges to overcome the limitations focus on fast and efficient de-adhesion mechanisms to increase speed and throughput in robotic operations. The combination of electrode adhesion with soft robotics, smart materials, and AI-driven control systems will expand applications to serve in industries and every day life.

This article traces the root of smart adhesive technology to its foundation in electrostatics. Socratic-style dialogue with ChatGPT revealed the interdisciplinary pathways through which scientific knowledge evolves over time beginning with the ancient observation of electrostatic effects in rubbed amber. The historical trajectory through landmark experiments and the evolution of material chemistry lead to advancing technologies of electrode adhesive smart materials and robotic grippers. While review and research articles were compiled from the Scopus database, ChatGPT uncovered reference sources beyond the journal indexing, which were subsequently retrieved and verified. This collaboration with LLM-powered chatbots demonstrates its role for language models in fostering historical awareness, interdisciplinary synthesis, and creative thinking in scientific development.

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During the preparation of this work the authors used ChatGPT (powered by OpenAI's language model, GPT-5) in order to identify conceptual connections and thematic continuities. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Research Article

Polyvalent Antivenom Potential of *Cnestis ferruginea* Root Extracts: Chromatographic Analysis and *In Vivo* Efficacy Against Snake Venoms

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Abstract. Snakebite is a major challenge in the world. Venoms of different snakes exert haemotoxic, neurotoxic and cytotoxic effects on the victims. Despite the successful use of serum therapy, antivenom has limitations especially in neutralizing tissue damage and being unavailable for use when needed. In this study, *Cnestis ferruginea* root extracts were investigated for antisnake venom properties against *Naja melanoleuca*, *Naja haje*, *Naja kateinsis* and *Echis ocellatus* venoms in albino rats. Concentrations used were found to have neutralized almost all toxic effects induced by the venoms. *In vivo* assessment of venom lethality (LD₅₀) for all four venoms was determined using probit analysis and found to be 0.455 and 0.465 mg/kg. The extracts neutralized the lethality of the venoms with effective dose at 50% survival (ED₅₀) found to be 1.4 mg/kg. Haematology and histopathology of liver and kidney of test rats were conducted to show the venom toxic effect and the ameliorative effect of the extract on envenomation. Phytochemical analysis of *C. ferruginea* root extract showed that it contains biochanin A, formononetin, genistein, anethole, myristicin, quercetin and chlorogenic acid which have antivenom properties. The root extracts have inhibitory action against hemorrhagic, inflammatory, and myotoxic activities of the venoms; and can be considered as polyvalent antivenom.

Keywords: Polyvalent, Antivenom, *Naja* spp., *Echis ocellatus*, *Cnestis ferruginea*, Snake bite

INTRODUCTION

Snakebites are injuries caused through bites from snakes, particularly venomous snakes, as a result of accidental provocation and are regarded as “neglected public health issue” worldwide. This prompted the World Health Organization, in 2017, to add snakebite envenoming to its list of highest priority neglected tropical diseases (NTDs).¹ World Health Organization estimated that over five million people are bitten annually, and approximately

45% develop one or more clinical illnesses. Additionally, the death count of such clinical illness ranges between 80,000 and 130,000 people annually.² Physiological process of snake envenoming is complex and includes the combined action of several toxins such as snake venom metalloproteinases (SVMPs), snake venom serine proteinases (SVSPs), phospholipases A2 (PLA2), hyaluronidases, bradykinin-releasing enzymes, lectins, L-amino oxidases, and pharmacological mediators.³

In Nigeria, a survey estimated that snakebites occur at 497 cases per 100,000 people; and out of this figure, 12% are killed while some have one arm or leg amputated to save their lives. Although two families of venomous snakes (Viperidae and Elapidae) have been reported to be associated with envenoming in Nigeria, the survey showed that the West African carpet viper (*Echis ocellatus*) is the species responsible for most bites (90%) and 66% of deaths.⁴

Echis cellatus are characterized by their bulging eyes and short snouts, a characteristic of the *Echis* genus. Its record length is between 30-50 cm.⁵ Its venom contains procoagulants, anticoagulants, haemorrhagins, nephrotoxins and necrotoxins. Its envenoming rate is estimated to be 80% and lethality not less than 20%.⁶ The vipers are a family of snakes found in various parts of the world except Antarctica, Australia, Hawaii, Madagascar, and some other isolated islands. They are oviparous but most are ovoviviparous. Their venom contains proteases (protein-degrading enzymes) that cause pain, disruption of the blood-clothing system, swelling and blood loss.

Naja melanoleuca, *Naja haje* and *Naja katiensis* are of the Elapid family, commonly known as cobras. They are oviparous, venomous, and characterized by their permanently erect fangs. Their venoms are mainly neurotoxic containing phospholipases A2 and three-finger toxins.⁵ Venom of *Naja melanoleuca* has been reported to have fifty-two different proteins, with three-finger toxins being the most abundant (57.1 wt.%) and 12.9 wt.% of phospholipases A2.⁷

Antivenom serum therapy administered intravenously has been the only specific treatment for snakebites. This antivenom, however, has some limitations such as excessive cost of purchase and unavailability, difficulty in storage, hypersensitivity reactions, exact dosage and close expiration which regulates its usage.⁸ Administration of the antivenom may prevent death but does not prevent local tissue damage and resultant disabilities. Due to the delay in receiving antivenom serum or its low efficacy, there is low inhibition of local effects which is the leading cause of amputations, that can lead to serious social, economic, and health negative impacts, given that most victims live in rural areas.⁹ Due to these

challenges, the search for new complementary therapies to treat snakebites gained importance. The use of medicinal plants for the treatment of envenomation is a longstanding practice, particularly in rural communities of Africa, Asia, and South America.⁹ *Cnestis ferruginea* is among the medicinal plants employed locally for the management of snakebites in the South-South region of Nigeria. This study was designed to assess the effectiveness of *Cnestis ferruginea* root as an inhibitor of snake venom toxins, aiming to validate or challenge its traditional use in snakebite management.

Cnestis ferruginea belonging to Connaraceae family is a secondary shrub with densely and rusty brown branches, with pear-shaped orange-red fruits. It is native to Africa and known to possess various antimicrobial properties.¹⁰ This plant is used in traditional medicine for a variety of purposes; as an aphrodisiac, enema for gynecological disorders, dysentery, urethral discharge and in the management of psychiatric disorders.¹¹ It is used against bronchitis; as laxative taken by the Yorubas in decoction. In Côte d'Ivoire, in cases of migraine & sinusitis (root); and conjunctivitis (fruit juice). For treatment of pyorrhea (powdered bark); against headache, as red dye by the Mende of Sierra Leone for dyeing clothes. It is used against toothache (rootbark paste) by Igbo of South-eastern Nigeria and Ghana. The stem is used by the Igbo in Southeastern Nigeria to make bows.¹²

This research explored the neutralizing activity of *Cnestis ferruginea* root extracts against cobras (*Naja melanoleuca*, *Naja haje* and *Naja katiensis*) and viper (*Echis ocellatus*) venoms using *in vivo* methods and antivenom compounds were identified using thin layer chromatography.

MATERIALS AND METHODS

Collection and Authentication of Plant Material

Fresh roots of *Cnestis ferruginea* were obtained by 10:30 hours on the 25th of May 2021 from Ipo forest in Ikwerre Local Government Area, Rivers State, Nigeria. The roots were identified and authenticated by Dr. M. G. Ajuru at the herbarium in the Department of Plant Science and Biotechnology, Rivers State University, with voucher number RSU Pb094. The Voucher specimen was deposited at the herbarium.

Preparation of Cnestis Ferruginea Root Extracts

The roots were rinsed of debris, cut into smaller sizes, air-dried, covered with muslin cloth to prevent dust

from resting on them and left for two weeks at room temperature (27 °C) until completely dehydrated (air-dried). The dried roots were ground into powder to create a larger surface area for maceration and subjected to sequential extraction using dichloromethane (DCM) and ethanol for 48 hours each. Each extract was decanted, filtered, and concentrated using a rotary evaporator at 40 °C to give the crude extracts. Distilled water was used to prepare the concentrations of extracts for administration.

Snake Venoms

Naja melanoleuca, *Naja haje*, *Naja katensis* and *Echis ocellatus* venoms were milked from each adult snake at 9:30 hours of 20th June 2021, with the help of Mr. S. Abdullahi, a snake charmer at Bioresources Development Centre (BIODEC), Odi, Bayelsa State, Nigeria, according to the method of Goswani *et al.*¹³ All four venoms were colourless except for *Echis ocellatus* that was golden-yellow. Stock solution (3.2 mg/kg) was prepared by dissolving each of the venoms in normal saline and serially diluted to 0.1, 0.2, 0.4 and 1.6 mg/kg. The venoms were preserved in a refrigerator (at 4 °C).

Experimental Animals

Albino rats weighing between 170 - 200 g were used for this experiment. They were purchased from the animal house of the Department of Pharmacy, University of Port Harcourt, Rivers State, Nigeria and housed in plastic cages of 40 x 25 x 20 cm dimensions under standard conditions. They were provided with grower pelletized feed and clean water; and acclimatized for two weeks prior to the experiment.

Acute Toxicity Check

The method of OECD/OCDE 425 was adopted. This method is called “an up-and-down procedure for acute toxicity testing”.¹⁴ Twenty-one rats were used; six groups of three rats each were orally administered with 5000, 4000 and 2000 mg/kg body weight doses of DCM and ethanol extracts, respectively, while the seventh group, which served as the control, was given an equivalent volume of distilled water. The rats were fasted overnight before administration and were observed for 24 hours after administration for toxic signs like excitability, dullness, diarrhea, oedema, and death.

Venom Median Lethal Dose Determination

The LD₅₀ (lethal dose at 50% death) of the venoms were determined using the method of Theakston and Reid.¹⁵ Sixty-three rats weighing between 170 - 200 g each were randomly distributed into twenty-one groups of three rats each. Group one (positive control) was given food and water only. Groups 2-6, 7-11, 12-16 and 17-21 were administered with varying concentrations (3.2, 1.6, 0.4, 0.2 and 0.1 mg/kg) of *Naja melanoleuca*, *Naja haje*, *Naja katensis* and *Echis ocellatus*, respectively. Mortality was recorded within 24 hours of venom administration and the LD₅₀ estimated using probit analysis. Some of the rats were dissected, organs and blood samples were collected and preserved for analysis.

Antivenom Assay

Experimental rats were divided into eleven groups of three rats each. Groups 1-11 were injected with 0.1 ml LD₅₀ of *N. melanoleuca* venom (0.455 mg/ml). While group one served as the negative control (envenomed group), groups 2-6 were intravenously injected with 400, 200, 100, 50, 25 mg/kg DCM extract, while 7-11 were injected with 400, 200, 100, 50, 25 mg/kg of the ethanol extract. The same analysis was conducted but with oral administration of extracts because of the outcome of the intravenous treatment. The rats fasted overnight prior to envenomation and treatment; observation was done for 24 hours and results were recorded.

Subsequently, extracts were further serially diluted to 12.5, 6.25 and 3.125 mg/kg. A new set of rats were divided into eight groups of three rats each. Group one served as the positive control (food and water only); groups 2-8 were injected with 0.1 ml LD₅₀ (0.455 mg/ml) of *N. melanoleuca* venom. Group 2 served as the negative control (envenomed group). Groups 3-5 were orally administered/treated with 12.5, 6.25 and 3.125 mg/kg DCM extract, while groups 6-8 were orally administered with 12.5, 6.25 and 3.125 mg/kg of ethanol extract, respectively. The lowest concentration of *C. ferruginea* extract with zero death (25 mg/kg) was taken as the ED₁₀₀ (effective dose at 100% survival).

Extended work was done to include the other three venoms using their various LD₅₀ (0.455 mg/ml and 0.465 mg/ml) and ED₁₀₀ of *C. ferruginea* extract (25 mg/kg). A separate set of rats were divided into thirteen groups of three rats each. Groups 1-3 were injected with 0.1 ml LD₅₀ (0.455 mg/ml) of *N. melanoleuca* venom, while groups 4-6, 7-9 and 10-12 were injected with 0.1 ml LD₅₀ of *N. haje*, *N. katensis* and *E. ocellatus* venoms, respectively. Groups 1, 4, 7, and 10 served as negative control

(envenomed group). Groups 2, 5, 8 and 11 were orally administered (treated) with 25 mg/kg DCM extract; while groups 3, 6, 9 and 12 were orally administered with 25 mg/kg ethanol extract. Group 13 was given food and water only (positive control). They were observed for 24 hours and results were recorded. Some of the rats were dissected, organs and blood samples (by jugular puncture) were collected and preserved for analysis.

Effective dose at 50% survival (ED_{50}), according to Spearman & Karber method, was calculated using Equation 1.¹⁶

$$ED_{50} = \log X_{100} - \log \frac{FD}{n} (\Sigma t - n/2) \quad (1)$$

Where: ED_{50} = the 50% effective dose; $\log X_{100}$ = log dose giving 100% survival (25 mg/kg); $\log FD$ = the log dilution factor (2); n = number of rats used at each dose level (3); Σ = the sum of rats surviving at every dose level; t = number of rats alive at each dose level (14)

$$ED_{50} = \log 25 - \log \frac{2}{3} \Sigma (14 - 3/2)$$

$$ED_{50} = 1.2979 - 0.1003 (12.5) = 0.144$$

$$ED_{50} = 100.144 (\text{Antilog}) = 1.393 \approx 1.4 \text{ mg/kg}$$

In order to assay the calculated ED_{50} , a new set of rats were divided into twelve groups of four rats each. Groups 1 - 4 served as the negative control (envenomed groups). Two groups each were consecutively injected with LD50 of *N. melanoleuca*, *N. haje*, *N. katiensis* and *E. ocellatus* venoms. One group each out of the two injected with each of the venoms were orally administered with 1.4 mg/kg (ED_{50}) of DCM extract, while the others were orally administered with 1.4 mg/kg of ethanol extract. They were observed for 24 hours and results were recorded. Some of the rats were dissected, organs and blood samples were collected and preserved for analysis.

Statistical Analysis

LD_{50} of venoms were determined using probit analysis. Data generated from haematology of test rats were presented as mean $\pm$ SD and subjected to one-way analysis of variance (ANOVA). Statistical difference between the means were compared using Student's T-test with the aid of a statistical package (IBM SPSS Statistics 20).

Thin Layer Chromatography and Phytochemical Analysis

Freshly prepared extracts were subjected to stand-

ard phytochemical analysis for various constituents such as isoflavone, phenylpropenes, glycosides, acidic compounds, anthraquinones, steroids, terpenoids.^{17, 18} Thin layer chromatography of the root extracts was conducted using standard methods.^{19, 20}

RESULTS

Extract Yield

DCM and ethanol extracts of *Cnestis ferruginea* yielded 16.60 g (0.33%) and 40.46 g (0.79%), respectively. DCM extract was greenish-brown sticky residue while the ethanol extract was reddish-rusty brown, like the colour of the fresh root. Both extracts had a fruity sweet aroma.

Toxicity of Extracts

Toxicity assay of DCM and ethanol extracts administered intravenously gave over 30% death rate for DCM and ethanol extracts (Figure 1). No death was recorded for oral administration except for DCM extract with 30% death rate for 5000 mg/kg body weight of rat (Figure 2) and no death for controls (administered food and water only). Both extracts significantly increased movement and smartness in animals (after oral administration) till the end of the study.

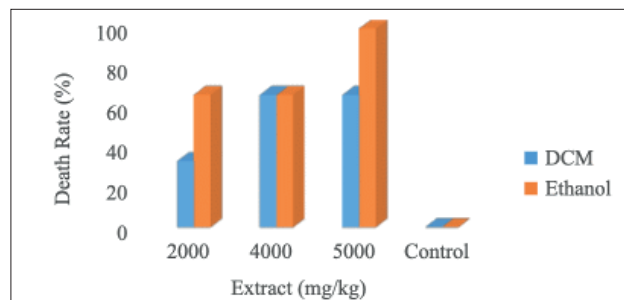


Figure 1. Dose response for acute toxicity of extracts showing death rates for DCM and ethanol extracts administered intravenously

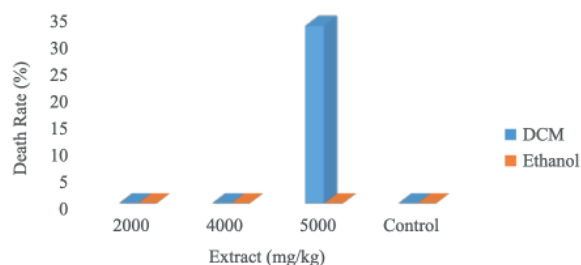


Figure 2. Dose response for acute toxicity of extracts showing death rates for DCM and ethanol extracts administered orally

Lethality and LD₅₀ of Venoms

After 24 hours post envenomation, the LD₅₀ for *N. melanoleuca* and *N. katiensis* was estimated to be 0.455 mg/kg while that of *N. haje* and *E. ocellatus* was 0.465mg/kg. Toxic signs noted in the rats when injected with the venom were respiratory problems with sneezing, stiffening of neck, weakness and then death within four-hour interval. Figure 3 shows death rate for each venom and control group (administered with food and water only).

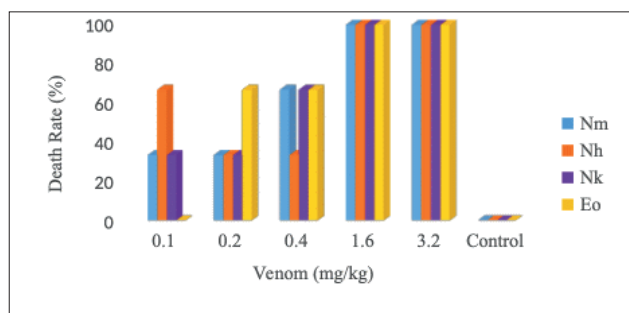


Figure 3. Lethality rate of venoms showing death rates for intravenous injection of *N. melanoleuca* (Nm), *N. haje* (Nh), *N. katiensis* (Nk) and *E. ocellatus* (Eo) venoms

Antivenom Activities of *Cnestis ferruginea* Extracts

Treatment for *N. melanoleuca* envenomation through intravenous injection of 25, 50, 100, 200 and 400 mg/kg DCM and ethanol extracts resulted to high death rate amongst the test group of organisms (Figure 4) but reduced mortality for oral administration (Figure 5). Death was first recorded for groups treated with ethanol extract, then for DCM extract after several hours. The least concentration that gave zero death for both extracts (oral administration) was 25 mg/kg; this was considered as ED₁₀₀ (Figure 5). Oral administration of ED₁₀₀ (25 mg/kg) to groups envenomed by all four venoms, resulted in the reduction of death rate to less than 30% except for groups injected with *N. katiensis* venom (Figure 6). ED₅₀ (1.4 mg/kg), when administered orally to envenomed animals, gave less than 40% death rate except for the group envenomed by *N. haje* (Figure 7). All positive control groups recorded zero death and all negative control groups recorded 100% death.

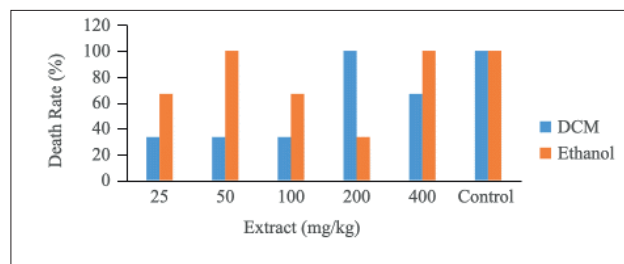


Figure 4. Dose-dependent response to intravenous treatment of *N. melanoleuca* envenomed rats with DCM and ethanol extracts at different concentrations

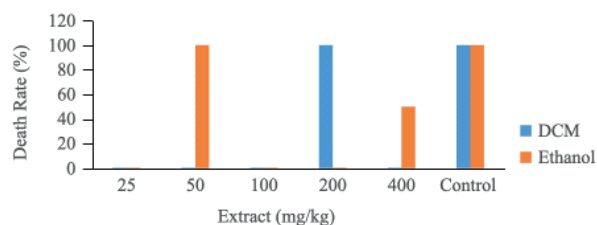


Figure 5. Dose-dependent response to oral treatment of *N. melanoleuca* envenomed rats with DCM and ethanol extracts at different concentrations

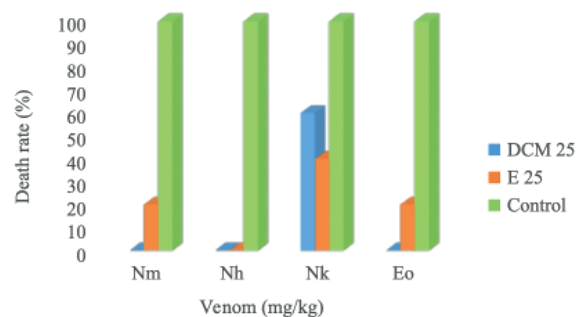


Figure 6. Treatment response of envenomed (by *N. melanoleuca* (Nm), *N. haje* (Nh), *N. katiensis* (Nk) and *E. ocellatus* (Eo) venoms) rats to ED₁₀₀ of DCM (DCM 25) and ethanol (E 25) extracts

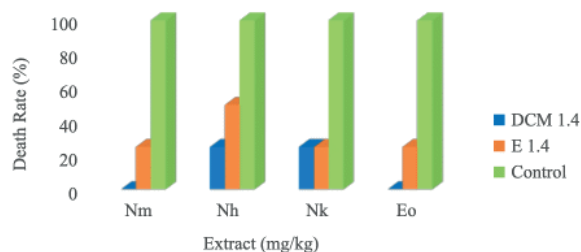


Figure 7. Treatment response of envenomed (by *N. melanoleuca* (Nm), *N. haje* (Nh), *N. katiensis* (Nk) and *E. ocellatus* (Eo) venoms) rats to ED₅₀ of DCM (DCM 1.4) and ethanol (E 1.4) extracts

Table 1. Haematological profile of control group (NØ), envenomed groups (Nk, Nm, Nh, and Eo), and extract treated groups (C1, C2 and G3)

Sample ID	AST (U/L)	ALT (U/L)	ALP (U/L)	T.B (µmol/l)	ALB (g/l)	K ⁺ (mmol/l)	Na ⁺ (mmol/l)	Cl ⁻ (mmol/l)	PCV (%)	RBC (x10 ¹² /l)	WBC (x10 ⁹ /l)	Ur (mmol/l)
Limit	0-35	3-15	44-147	1.71-20	35-53	3.6-5.2	135-145	96-106	40-53	4.2-5.4	4.5-11	2.1-8.5
NØ	26±1.73	6.6±0.36	91±4.04	5.7±0.46	38±1.00	3.5±0.12	136±1.73	63±7.21	49±2.31	4.10±0.62	7.1±0.62	4.6±0.32
C1	25±1.73	6.1±0.15	80±2.08	5.5±0.56	38±0.58	3.1±0.35	146±2.65	63±1.73	45±2.65	4.9±0.26	7.4±0.55	3.7±0.26
C2	29±6.03	6.8±0.30	110±2.65	6.3±0.44	35±1.73	3.2±0.26	140±4.36	69±2.65	43±3.61	5.5±0.36	7.7±0.36	3.5±0.52
G3	23±4.36	7.5±0.36	98±4.58	4.9±0.31	38±1.00	3.5±0.10	137±2.65	62±3.61	46±2.08	5.9±0.44	7.8±0.26	3.3±0.36
Nk	39±3.61	9.1±0.30	85±3.61	14.2±0.36	32±1.53	2.5±0.46	115±3.61	65±3.61	34±2.65	3.9±0.36	11.6±0.62	6.5±0.17
Nm	40±2.65	9.3±0.26	92±4.93	14.5±0.52	32±2.65	2.8±0.36	112±3.61	81±3.51	38±2.08	3.7±0.42	11.9±0.46	6.2±0.40
Nh	38±1.53	10.8±0.17	100±8.62	14.3±0.46	33±1.15	2.7±0.17	129±1.73	72±4.73	34±2.65	3.7±0.17	12.5±0.26	8.3±0.26
Eo	49±5.29	16.8±0.30	83±4.73	18.5±0.44	30±2.65	2.2±0.56	110±0.58	87.7±2.52	28±2.65	3.4±0.26	13.3±0.44	8.8±0.10

Mean ± standard deviation differs from each other with 95% confidence interval. NØ = positive/ control group; C1 = *Echis ocellatus* venom + 1.4 mg/kg DCM group; C2 = *Echis ocellatus* venom + 1.4 mg/kg ethanol group; G3 = *Echis ocellatus* venom + 25 mg/kg DCM group; ALP = Alkaline Phosphatase; ALT = Alanine Aminotransferase; AST = Aspartate AminoTransferase; µmol/l = micro moles per liter; mmol/l = millimoles per liter; PCV = Packed Cell Volume; RBC = Red Blood Cell; WBC = White Blood Cell; Nm = *Naja melanoleuca* envenomed group; Nh = *Naja haje* envenomed group; Nk = *Naja katiensis* envenomed group; Eo = *Echis ocellatus* venom group; TB = Total Bilirubin; U/L = units per liter; Na⁺ = Sodium; Cl⁻ = Chloride; Ur = Urea; g/l = gram per liter; K⁺ = Potassium; ALB = Albumin.

Haematological Profile of Test Animals

Table 1 shows the results of haematological analyses of collected blood samples. The results show variations in parameters for positive control, negative controls and groups treated with DCM and ethanol extracts after envenomation. AST, T.B and ALB for the normal group (NØ) and treated groups (C1, C2, G3) were within limit compared to the envenomed groups (Nm, Nh, Nk and Eo). ALT and ALP values showed no significant differences between normal, treated, and envenomed groups except for ALT in Eo group. The electrolyte panel test (K⁺, Cl⁻ and Na⁺) showed significant differences ($p < 0.005$) between the normal and treated groups compared to envenomed groups (Nm, Nh, Nk and Eo). Urea (Ur), PCV and RBC were higher in control (NØ) and treated groups (C1, C2, G3) than in envenomed groups (Nm, Nh, Nk and Eo). White blood cells (WBC) were lower in normal (NØ) and treated groups (C1, C2, G3) than in envenomed groups (Nm, Nh, Nk and Eo).

Histopathological Profile of Liver

Results of the effects of *C. ferruginea* DCM and ethanol extracts on cellular necrosis induced by *N. melanoleuca*, *N. haje*, *N. katiensis* and *E. ocellatus* venoms on liver compared to normal group show pathological differences in the effect of the venom and extract treatment. The micrograph of liver for positive control group (Figure 8) shows a healthy non-distorted liver but those of the envenomed groups (Figure 9) show distorted cells

due to the effects of the venoms. Micrographs of envenomed liver treated with ED₁₀₀ of the extract shows cells healed from distortion caused by venom (Figure 10); while those of groups treated with ED₅₀ of the extracts (Figure 11) show that the distorted cells have been healed from the distortion caused by the venom.

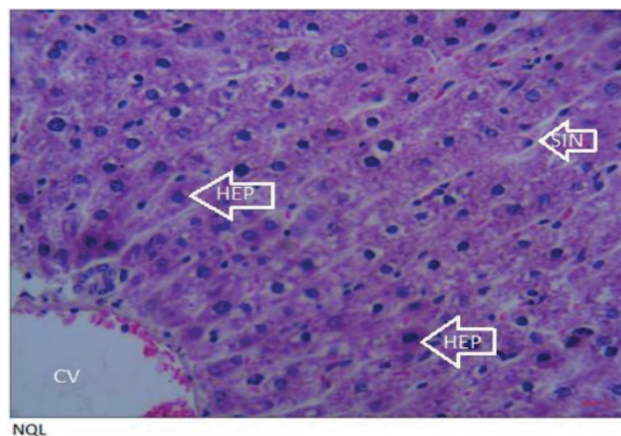


Figure 8. Liver of positive control group (NQL) showing histologically normal liver with patent central vein (CV), intact hepatocytes (HEP), and sinusoid (SIN) containing capillaries and Kupffer cells

Histopathological Profile of Kidney

Results of *C. ferruginea* DCM and ethanol extracts on envenomation induced by *N. melanoleuca*, *N. haje*, *N. katiensis* and *E. ocellatus* venoms on kidney in comparison with positive control group (normal group) and extract treated groups showed pathological differences in

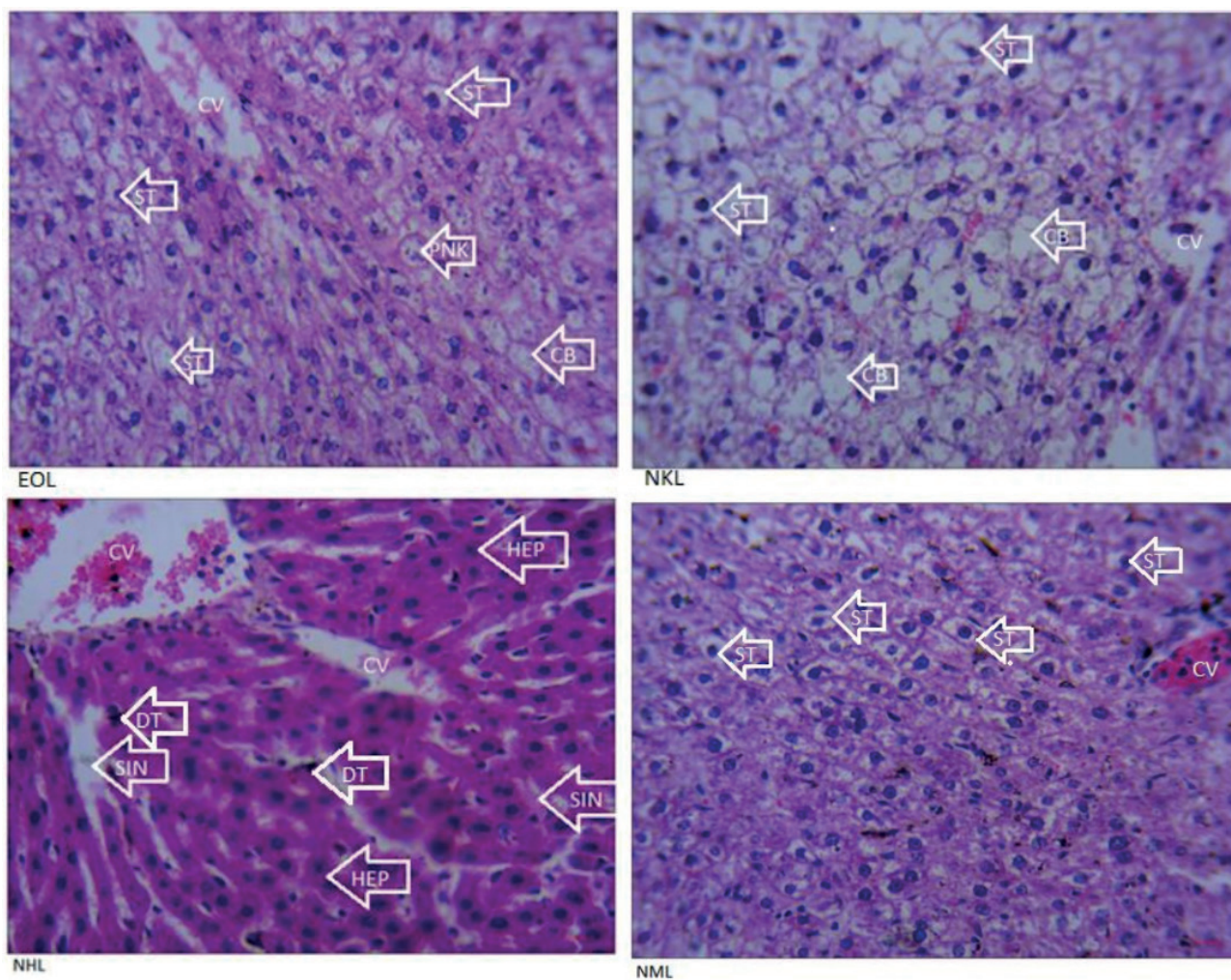


Figure 9. Liver of envenomed groups (EOL = *E. ocellatus* envenomed liver; NKL = *N. katiensis* envenomed liver; NHL = *N. haje* envenomed liver; NML = *N. melanoleuca* envenomed liver) showing histologically distorted tissues (DT), with various levels of fatty changes (ST = steatosis) and Councilman's body (CB), patent central vein (CV), sparsely distributed normal hepatocytes (HEP), and nuclear disintegration (PNK = Pyknosis)

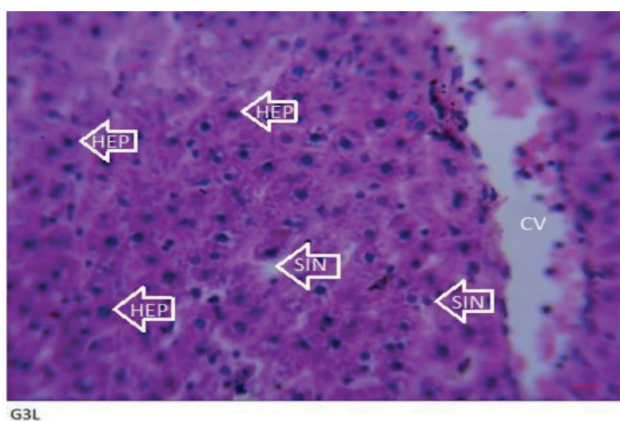


Figure 10. Liver of group treated with ED₁₀₀ of DCM extract (G3L) showing normal patent central vein (CV), intact hepatocyte (HEP), sinusoid (SIN) containing capillaries and Kupffer cells

venom toxicity and extract treatment. The micrograph of kidney for positive control group (normal group) (Figure 12) shows a healthy non-distorted kidney but those of the envenomed groups show distorted cells due to the effects of the venoms (Figure 13). Micrograph of envenomed kidney treated with ED₁₀₀ of DCM extract shows cells healed from distortion caused by venom (Figure 14). Groups treated with ED₅₀ of DCM extract (Figure 15) and ethanol extract (Figure 16) showed the distorted cells being healed from distortion caused by venom.

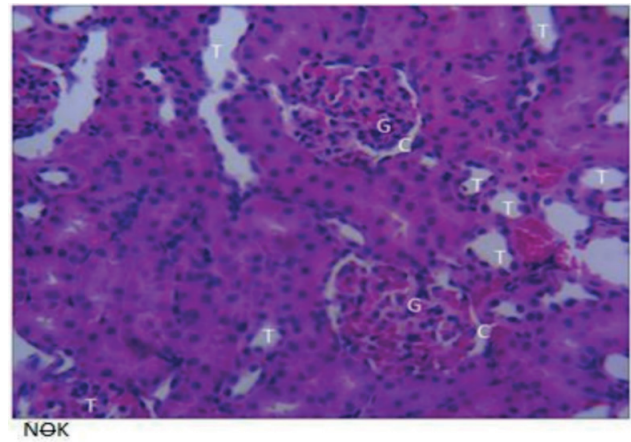
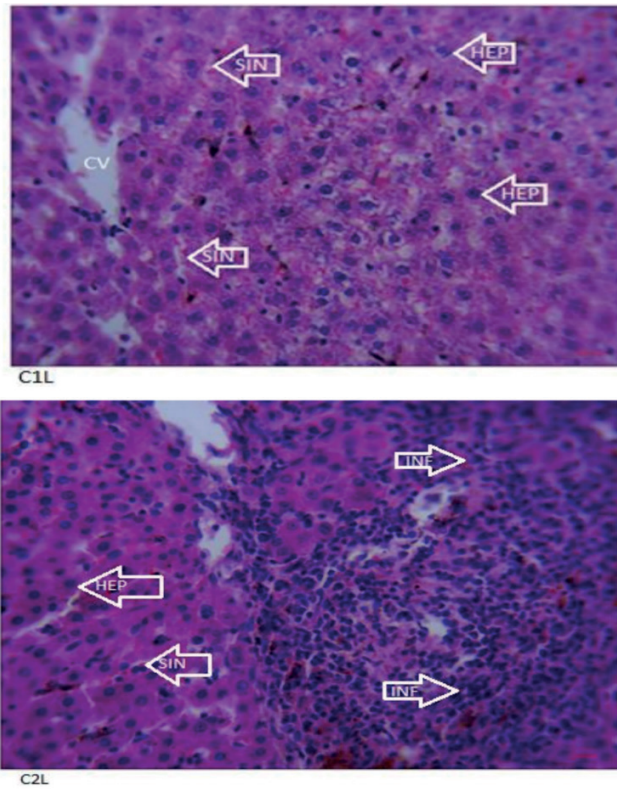


Figure 12. Kidney of positive control group showing histologically normal Kidney with glomeruli (G) surrounded by patent Bowman's Capsular (C) spaces and Tubules (T) lined by simple epithelia.

Figure 11. Liver of groups treated with ED_{50} of DCM (C1L) and ethanol (C2L) extracts showing normal and sparsely conjugated patent central vein (CV), intact hepatocyte (HEP), inflammation (INF) gradually tending to normal, and sinusoid (SIN) containing capillaries and Kupffer cells

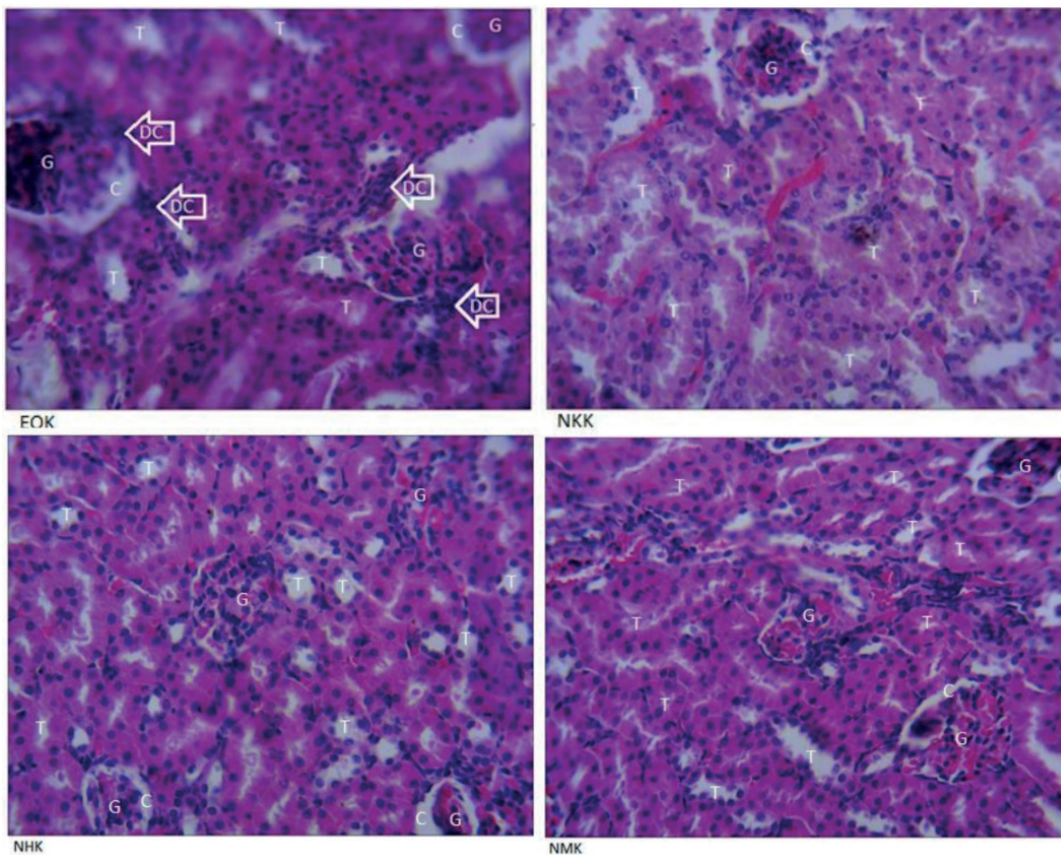
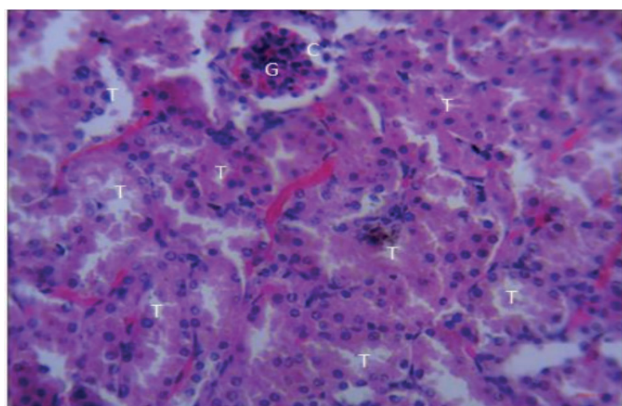
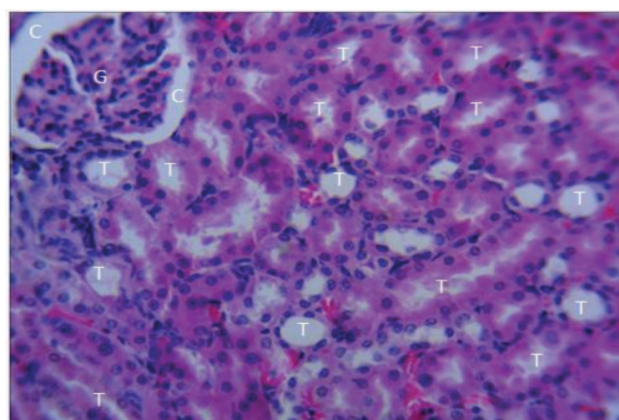
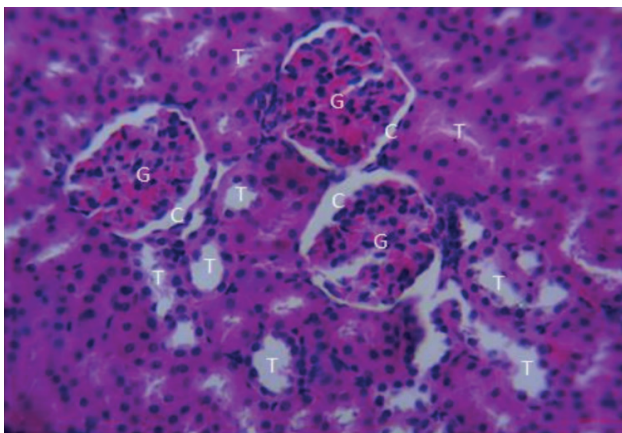


Figure 13. Kidney of envenomed groups (EOK = *E. ocellatus* envenomed kidney; NKK = *N. katiensis* envenomed kidney; NHK = *N. haje* envenomed kidney; NMK = *N. melano-leuca* envenomed kidney) showing distorted cells (DC), glomeruli (G) surrounded by patent Bowman's capsula (C) spaces, tubules (T) lined by simple epithelia

Table 2. Activities of compounds identified from the extract of *Cnestis ferruginea* root

S/No.	Phytochemical	Phytochemical class	R _f value (experimental)	R _f value (literature)	Activity against snake venom
1	Biochanin A	Isoflavones	0.88	0.88	Anti-inflammatory, induces relaxation of the coronary artery, neuroprotective, L-amino-acid oxidase inhibitor, phosphodiesterase inhibitor. ¹⁹
2	Formononetin	Isoflavones	0.90	0.85	Anti-inflammatory, phosphodiesterase inhibitor, neuroprotective, antioxidant, osteogenic action. ¹⁹
3	Genistein	Isoflavones	0.80	0.83	Anti-inflammatory, inhibition of the nicotinic acetylcholine receptor. ¹⁹
4	Anethole	Phenylpropenes	0.68	0.69	Inhibits phosphodiesterase, anti-inflammatory, inhibits ATPase, inhibits monoamine oxidase. ²⁰
5	Myristicin	Phenylpropenes	0.55	0.50	Inhibits ATPase, inhibits monoamine oxidase, anti-inflammatory, hepatoprotective, inhibits phospholipase A2. ²⁰
6	Quercetin	Terpenoids	0.45	0.43	Antidote, inhibits snake venom phospholipase A2, inhibits 5'-nucleotidase, inhibits ATPase, phosphodiesterase inhibitor. ²¹
7	Chlorogenic acid	Polyphenol esters	0.65	0.64	Anti-inflammatory, ATPase inhibitor, inhibits 5'-nucleotidase, phospholipase inhibitor, antidote. ²¹

**G3K****Figure 14.** Kidney of group treated with ED₁₀₀ of DCM extract (G3K) showing normal healthier glomeruli (G) surrounded by patent Bowman's Capsular (C) spaces and Tubules (T)**C2K****Figure 16.** Kidney treated with ED₅₀ of ethanol extract (C2K) showing healthy normal kidney containing glomeruli (G) surrounded by patent Bowman's capsular (C) spaces and Tubules (T) because of successful extract treatment**C1K****Figure 15.** Kidney of group treated with ED₅₀ of DCM extract (C1K) showing healthy normal kidney containing glomeruli (G) surrounded by patent Bowman's Capsular (C) spaces and Tubules (T)

Phytochemicals with antivenom activity identified in Cnestis ferruginea Root Extract

Seven compounds made up of isoflavones, phenylpropenes, terpenoids and polyphenolic esters were identified in the root extract of *C. ferruginea* via thin layer chromatography. Biological activities of the identified compounds against snake venoms are as shown in Table 2.

DISCUSSION

Antiphidic ability of *Cnestis ferruginea* root extracts was investigated, in order to propose alternative options for the management of envenomation, by using a

plant that is known locally for treatment of snake-related bites in southern Nigeria. Acute therapeutic mode of treatment was adopted since most treatments occur after a bite. This study and many others confirm the ability of *N. melanoleuca*, *N. haje*, *N. katiensis* and *E. ocellatus* venoms injection to induce muscle damage, edematogenic activity, hyperalgesia, and motor function impairment. Several metalloproteinases and phospholipases A2 (PLA2) are among the important components of venoms that present cytotoxic and inflammatory properties.²²

Extraction of *C. ferruginea* root using DCM and ethanol yielded 16.6 g (0.79%) and 40.6 g (0.33%), respectively, though Sivaraman *et al.* reported a 2.5% yield from 20 g powdered root sample.²³

DCM and ethanol extracts of *C. ferruginea* root increased movement and smartness in the animals when used alone. For intraperitoneal injection, the acute toxicity result with 2000, 4000 & 5000 mg/kg showed mortality rate of over 50% in all the groups except 2000 mg/kg DCM extract group with less than 40% death rate (Figure 1), but for oral administration, no mortality was observed within the experimental groups after 24 hours post administration of the same concentrations of both extracts (2000, 4000 & 5000 mg/kg) except for 5000 mg/kg DCM extract (Figure 2). The animals remained active and survived for up to 28 days, indicating that the administered doses of the extract were insufficient to induce toxicity. Notably, the rats exhibited increased movement and activity compared to their behavior prior to receiving the extract. Consistent with these findings, it can be concluded that *C. ferruginea* root extracts are nontoxic when administered orally. Therefore, the extract may be considered safe for use as a potential medication. This result is in line with the acute toxicity work done by Enenebeaku *et al.*²⁴ Ishola *et al.* also stated that no death was recorded at doses of 1 and 2 g/kg but animals were calm, hypoactive, and passed watery stools.¹¹ It was also noted that intraperitoneal administration proved to be more toxic compared to oral administration. This increased toxicity may be attributed to a possible infection at the site of administration. Additionally, intraperitoneal administration allows the substance to enter the bloodstream more rapidly and directly, bypassing the liver. In contrast, oral administration requires the substance to pass through the liver first, where it is often metabolized and weakened before reaching systemic circulation.

Lethality at 50% death using 0.1, 0.2, 0.4, 1.6 and 3.2 mg/kg body weight gave LD₅₀ of 0.455 mg/ml for *N. melanoleuca* and *N. katiensis*; 0.465 mg/ml for *N. haje* and *E. ocellatus* using probit analysis. These lethal doses correspond with LD₅₀ of *N. nigricollis* (cobra) and *E.*

carinatus (viper) as reported by Ernst and Zug.²⁵ Phospholipase A2 (PLA2) is the most toxic component of the venom and responsible for wide range of pharmacological problems.¹³ Venom toxicity signs noted in the rats were respiratory problems with sneezing, stiffening of neck, weakness and then death within four-hour interval. This result tallies with the result of Ranawaka *et al.* that elapids (cobra) are mainly neurotoxic.²⁶ Death rate is as shown in Figure 3.

Effectiveness of the extracts against the venoms are described in terms of comparison of prevention of deaths and improvement in health of extract-treated groups with that of envenomed groups (negative controls). Extract intravenous treatment of envenomation, for groups treated with 25, 50, 100, 200 and 400 mg/kg of DCM and ethanol extracts, respectively, after envenomation with *N. melanoleuca*, gave over 50% death for most of the groups (Figure 4), which may be as a result of arterial or blood vessels hit and/or site infection at the site of injection but none for oral administration except for DCM 200 mg/kg, ethanol 50 and 400 mg/kg groups (Figure 5).²⁷

Death rate for groups orally treated with ED₁₀₀ (25 mg/kg) of DCM and ethanol extracts, after envenomation (using all four venoms: *N. melanoleuca*, *N. haje*, *N. katiensis* and *E. ocellatus*), showed less than 25% death rate except for *N. katiensis* that indicated above 30% death rate for both extracts (Figure 6). The percentage survival (<50%) of envenomed rats administered with *C. ferruginea* appeared to be better with ED₁₀₀ (25 mg/kg) compared to ED₅₀ of 50 and 300 mg/kg reported by Iful for aqueous extracts of *Paullinia pinnata* and *Detarium microcarpum*, respectively.¹⁷

Result of administration of ED₅₀ (1.4 mg/kg) for each of DCM and ethanol extracts indicated less than 40% mortality rate except for *N. haje* envenomed group, which showed over 40% mortality for ethanol extract, after 24 hours (Figure 7). All envenomed groups treated with ethanol extract had higher mortality rate than the DCM extract-treated groups. This result showed better antivenom effectiveness than the ED₅₀ of 477 mg/kg reported by Aguilar *et al.*¹⁶

Results of oral treatments showed that the effect of the extracts on envenomed rats were better than intravenous administration. This is in line with the observation of Iful, who posited that oral treatment has better therapeutic effect than intravenous administration.¹⁷ Antivenom property of the extracts are in accordance with the findings of Fattepur and Gawade.²⁸

Improved health of the envenomed test rats after administration of the extracts showed the antivenom properties of the extracts as can be seen in their haematological (Table I) and histological results.

Significant reduction ($p < 0.005$) in ALT levels of extract treated groups (C1, C2, G3) as against envenomed groups were observed. Extract treated groups showed a much more reduced levels of ALT. Abnormal (high) levels of ALT were as a result of damaged or inflamed cells and are associated with conditions, such as inflammation (hepatitis) and scarring (cirrhosis), because it is most concentrated in and affects the liver.²⁹

Levels of AST in both DCM and ethanol treated groups reduced significantly ($p < 0.005$) compared to envenomed groups (Table 1). This study shows that the venoms increased capillary permeability in the rats, which was reversed by *C. ferruginea* root extracts.

ALP concentration in the bloodstream is used by diagnosticians as a biomarker in determining hepatitis or osteomalacia.³⁰ Abnormal levels of alkaline phosphatase in the blood of envenomed rat indicated issues relating to the liver, gall bladder or bones. There was more stable ALP levels in the groups treated with the extracts.

Total bilirubin (TB) was higher in envenomed groups, especially group induced with viper (Eo) compared to treated groups (C1, C2, G3) (Table 1). A high amount of bilirubin is expected of sickled or inflamed cells. The result shows that the extracts gave ameliorative response to the effect of the venom on the bilirubin level in the rats. Low albumin levels indicate a problem with the kidney, liver, or other health challenges. Liver panel includes measurements of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, and bilirubin (Table 1).²⁹

Increase in packed cell volume (PCV) in *C. ferruginea* treated rats compared to envenomed groups (Table 1), may be because of dehydration due to diuretic outcome of the extract.³¹ Leukocytosis (high white blood cell counts) occurred in envenomed rat groups, possibly due to tissue necrosis and inflammatory responses by the venom. High white blood cell count is the initial finding that leads to diagnosis of primary haematological disorder, like leukemia, in animals. The extracts eliminated these effects partly due to anti-inflammatory effects of the extract as described by Ishola *et al.*¹¹ The overall result indicated that the extracts of *C. ferruginea* root were protective of the blood cells against adverse effects of the snake venoms.

Electrolyte panel test levels (potassium, sodium, and chloride) were within range in the normal and treated groups compared to the significant difference in envenomed groups. Lowest value was observed in viper envenomed group (Eo). Potassium levels are used to monitor health conditions related to kidney disease. Potassium (K^+) levels were higher in envenomed groups compared

to extract-treated groups. Too much or too little levels of potassium in the blood indicates serious medical problems. Low sodium (Na^+) levels in envenomed groups (Nk, Nm, Nh, and Eo) are indications of kidney failure, as against the levels in extract-treated groups. Elevated levels of chloride as seen in envenomed groups are signs of dehydration and kidney disease, though there is a lower level in Nk envenomed group (Table 1).

Red blood cells or erythrocytes were lower in envenomed groups compared to extract-treated groups (Table 1). This is a result of an infected bone marrow, as red blood cells are produced there. The level of blood urea was within range for all groups except Eo group. Envenomed groups have higher urea values tending towards uremia, a dangerous medical condition that causes urea to accumulate in the blood because kidney could not filter it as waste, but it then enters the bloodstream. This shows the negative effect the venom has on the kidney; it also shows that the extracts ameliorated this effect in the rats and agrees with the work of Okafor *et al.*³²

Histopathological examination of liver sections in the positive control group (Figure 8) showed a patent normal appearance of central vein (CV) and intact hepatocytes and sinusoids (SIN) containing capillaries and Kupffer cells with no visible lesion compared to liver of envenomed groups (NML, NHL, NKL and EOL of Figure 9) with visible haemorrhages, distorted tissues, congested central vein, hepatocytes with different fatty changes (steatosis), councilman body and nuclear disintegrated pyknosis which are evidence of distortion. This coincides with the work of Adeyi *et al.*³³

The group treated with ED₁₀₀ (25 mg/kg) of DCM extract (Figure 10) showed histologically normal liver with patent central vein. Groups separately treated with ED₅₀ (1.4 mg/kg) of DCM and ethanol extract (Figure 11) also gave comparable results with that of the normal group. These are clear indications of a healthy liver due to treatment with the extracts.

The kidneys are a pair of bean-shaped organs on either side of the spine that filter blood, remove waste, control the body's fluid balance, and keep the right levels of electrolytes. Studies of the kidney sections of rats treated with ED₁₀₀ (Figure 14) and ED₅₀ (Figures 15 & 16) of both extracts showed no significant microscopic changes compared to those of the positive control (Figure 12). For these treated groups, kidney sections showed normal glomerulus (G) containing mesangial cells, mesangial matrix, and capillaries; normal Bowman's capsule spaces (C), renal tubules (T) lined with simple epithets. Compared to these are the envenomed groups (Figure 13), especially the viper venom, EoK, with distorted kidney showing damaged cells (DC).

This shows that the Glomeruli matrix; which contains the mesangial cells (blue/black), mesangial matrix/fluid (purple) and the capillaries (reddish) had been negatively affected by the presence of the venom; an indication of an unhealthy system. This agrees with the findings of Chaibabutr *et al.* that viper venoms affect the histopathology of kidney.³⁴ Extracts of *C. ferruginea* roots reversed the negative effects of the venom on the kidneys. This agrees with the conclusion made by Adeyi *et al.* that plants contain phytochemicals active against the negative effects of snake venoms.³³

Phytochemical analysis of *C. ferruginea* root extracts identified the presence of terpenoids, acidic compounds, polyphenol esters (alkaloidal), isoflavones and phenylpropenes (Table 2). Phytochemicals are usually involved in protection of various diseases and in curing. Functions of alkaloids are mostly related to protection. They are widely used as an analgesic, inhibitor of acetylcholinesterase.³⁵

The result of phytochemical analysis of *C. ferruginea* root extracts, revealed the presence of polyphenolic compounds (terpenoids, isoflavones and polyphenol esters), which are responsible for the neutralizing effect of plants that are in popular use against the action of snake venoms. This also suggests that *C. ferruginea* root extract have anti-snake venom potential since polyphenols possess protein-binding and enzyme inhibitory properties which inhibit snake venom phospholipase A2 (an enzyme present in cobra venoms) activities.^{36, 37} Phytochemicals counter venom-induced Phospholipase A2 (PLA2) activity primarily by binding to the enzyme's active site, blocking its ability to hydrolyze cell membrane phospholipids, and neutralizing its inflammatory effects. These plant-derived compounds can inhibit PLA2 enzymes, reducing venom-induced hemolysis, edema, and anticoagulation.

Phytochemical analysis of *C. ferruginea* carried out by Akharaiyi *et al.* showed that it contains tannins, alkaloids, saponins, phlobotannins, flavonoids, cardiac glycosides, anthraquinones and terpenoids; though Khalid *et al.* reported that aristolochic acid, a gymnemic acid, wedelolactone, cabenegrin-i, stigmastrol, sitosterol, ar-turmerone, rutin, hesperidin, iridin, caffeic acid, 2-hydroxy-4-methoxy benzoic acid are some phytochemicals that are active against snake envenomation.^{10, 38}

These results suggest that the inhibitory effect of the extracts against snake venom-induced pathological symptoms could be, partly, due to the active components of the phytochemical compounds and the likelihood of the extracts acting through a systematic intervention rather than through physical interface with the venom, which is the likely means of action of many polyphenolic compounds found in plant extracts.³⁹

Table 2 shows the activities of some phytochemicals present in the extracts. Isoflavones (biochanin A, formononetin and genistein) with R_f values of 0.88, 0.90 and 0.80 had similar value with that obtained by Wang.¹⁹ These isoflavones also exert various activities against snake venoms by inhibiting snake venom enzymes (phosphodiesterase, ATPase). Biochanin A is an O-methylated isoflavone recognized for its capability to counteract the toxicity of snake venom. Its protective effects are attributed to its chemical structure, which features both hydroxy and methoxy groups characteristic of phenolic compounds. The primary mechanism by which biochanin A mitigates snake venom toxicity involves the direct inhibition of venom enzymatic activity. Biochanin A acts as a small-molecule inhibitor, binding to the active sites of venom proteins. This interaction prevents the venom enzymes from engaging with target tissues and exerting their harmful effects. In addition to enzymatic inhibition, biochanin A contributes to the structural stabilization of venom proteins. By binding to these proteins, it helps maintain their structure, reducing their ability to interact deleteriously with biological tissues and further minimizing the toxic impact of the venom.⁴⁰ Phenylpropenes (myristicin and anethole) also showed similar R_f values (0.55 and 0.68, respectively) compared to that reported in literature and research showed they exert some activity against the effect of snake venom enzymes.²⁰ Myristicin is recognized in literature for its ability to inhibit phospholipase A2 (PLA2) enzymatic activity. This inhibition typically occurs through the binding of myristicin to the enzyme, which alters its structural integrity and interferes with its function.⁴¹ The terpenoids (quercetin and chlorogenic acid) similar to that obtained by Ćetković have been proven to be antidotes for snake bite (vipers and cobras).²¹ The terpenoids (quercetin and chlorogenic acid) similar to that obtained by Ćetković have been proven to be antidotes for snake bite (vipers and cobras).²¹

CONCLUSIONS

This study investigated the effects of snake venom on albino rats and the protective role of *Cnestis ferruginea* root extracts. Envenomed groups exhibited significant disturbances in electrolyte balance, red blood cell count, and kidney and liver structure, indicating organ damage. However, treatment with *Cnestis ferruginea* root extracts ameliorated these effects, resulting in near-normal test results and tissue health. Chromatographic analysis revealed the presence of various bioactive compounds such as terpenoids, isoflavones, and

polyphenols, which are proposed to contribute to the anti-venom properties by inhibiting venom enzymes. The findings suggest that *Cnestis ferruginea* root extracts have potential therapeutic value against snakebite-induced damage. The study not only demonstrates the therapeutic promise of *Cnestis ferruginea* root extracts in mitigating snakebite-induced organ damage but also contributes compelling evidence for the continued exploration and development of plant-based antidotes. Such therapeutic agents could play a crucial role in regions where snakebite envenomation remains a persistent medical challenge and access to conventional antivenoms is limited.

ETHICAL APPROVAL

Experiments were approved by the University of Port Harcourt Research Ethics Committee with assigned number UI-ACUREC: 18/0108, in accordance with the guidelines of the National Code for Health Research Ethics (NCHRE). All animal experiments were performed according to ethical standards.

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Research article

Modulating the AGE-RAGE Signaling Pathway in Degenerative Processes: Molecular Mechanisms and Therapeutic Prospect of Phytochemicals

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Abstract. The Receptor for Advanced Glycation End Products (RAGE) is a multi-functional cell-surface receptor capable of binding a broad range of ligands, including advanced glycation end products (AGEs). While RAGE is constitutively expressed in various tissues, AGEs arise from non-enzymatic reactions between reducing sugars and proteins, lipids, or nucleic acids. The AGE-RAGE interaction activates multiple redox-sensitive and pro-inflammatory signaling pathways, thereby contributing significantly to ageing, chronic inflammatory conditions, and metabolic degenerative diseases. Consequently, targeting the AGE-RAGE axis has become an important focus of contemporary biomedical research. Although substantial progress has been made in elucidating this pathway and developing synthetic AGE-RAGE inhibitors, many of these agents present limitations related to safety, bioavailability, or therapeutic efficacy. Phytochemicals such as polyphenols, alkaloids, and tannins have exhibited promising anti-glycation, antioxidant, and anti-inflammatory activities that may attenuate AGE-RAGE mediated cellular dysfunction. However, direct evidence of their capacity to inhibit RAGE at the receptor level remains limited. This review identifies critical knowledge gaps and demonstrates that structure-guided computational modeling combined with targeted experimental validation represents a promising strategy for the rational development of phytochemical-based RAGE modulators. These findings highlight the potential of natural products as safer and mechanistically informed therapeutic candidates for attenuating AGE-RAGE-mediated degenerative disease progression.

Keywords: AGE, RAGE, AGE-RAGE signaling, Ageing, Degenerative diseases, Phytochemicals

INTRODUCTION

Ageing is a progressive process characterized by the gradual accumulation of chemical modifications and structural damage to biological macromolecules such as DNA, proteins, and lipids, leading to an irreversible decline in cellular and physiological functions (Zhang et al., 2025). This decline is primarily driven

by molecular damage and imbalances in the body's homeostatic mechanisms, often influenced by endogenous and exogenous chemical factors (Choi et al., 2025). The chemistry of ageing is strongly associated with the free radical theory and the formation of advanced glycation end-products (AGEs) (Uceda et al., 2024a). Oxidative stress represents one of the most critical biochemical features of ageing. It results from the excessive production of reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals, and hydrogen peroxide by-products of normal cellular respiration (Dash et al., 2025). This process is further enhanced in the presence of transition metals like iron and copper through Fenton-type reactions (Song et al., 2024a). With advancing age, the body's natural antioxidant systems, including glutathione, superoxide dismutase, and catalase, become less efficient, thereby intensifying oxidative stress (Jomova et al., 2023). Consequently, oxidation of deoxyribonucleic acid (DNA) (e.g., formation of 8-oxo-dG), proteins, and lipids occurs, producing aldehydic by-products such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE), which can form covalent adducts with proteins and nucleic acids, leading to molecular dysfunction (Misra et al., 2025; Blouin and Saini, 2024).

The fundamental chemical interactions between ROS and biological macromolecules are central to cellular oxidative chemistry (Piras et al., 2016). During mitochondrial respiration, electrons escaping from the electron transport chain can react with oxygen to generate superoxide radicals (Napolitano et al., 2021). These radicals undergo dismutation to yield hydrogen peroxide, which in the presence of transition metals, produces highly reactive hydroxyl radicals via the Fenton reaction (Kanti Das., 2014). These radicals initiate lipid peroxidation by attacking polyunsaturated fatty acids in cell membranes, producing lipid radicals and hydroperoxides (Huang and Yang 2021). The subsequent decomposition of these hydroperoxides yields reactive aldehydes capable of cross-linking proteins and nucleic acids, thereby disrupting cellular integrity (Feng et al., 2024). Another major biochemical contributor to ageing is non-enzymatic glycation, a reaction between reducing sugars and free amino groups of lysine or arginine residues in proteins (Senatus and Schmidt, 2017). This process leads to the formation of unstable Schiff bases and Amadori intermediates that eventually rearrange into AGEs (Chen et al., 2024). AGEs impair biological function by inducing irreversible cross-links in long-lived structural proteins such as collagen, thereby decreasing tissue elasticity and resilience. Moreover, AGEs interact with the receptor for advanced glycation end-products (RAGE), a multi-ligand receptor that triggers oxidative stress and pro-inflammatory signaling cascades (Byun et al., 2017). RAGE activation also

stimulates matrix metalloproteinases (MMPs), enzymes that degrade the extracellular matrix, accelerating tissue damage and skin ageing (Feng et al., 2024).

Although ROS generation and AGE formation originate from distinct biochemical processes, they are closely interconnected. ROS promote glycation by facilitating oxidative conversion of Amadori intermediates into AGEs, a process known as glycooxidation (Pathomthongtawechai and Chutipongtanate, 2020). Consequently, AGE formation, RAGE activation, and ROS generation form a self-propagating feedback loop that amplifies cellular and tissue ageing (Kinscherf and Pehar, 2022). The AGE-RAGE axis constitutes a crucial point of convergence through which chemical damage is converted into persistent biological signaling. A comprehensive synthesis of current knowledge on RAGE is therefore necessary to elucidate its role as a molecular amplifier of ageing-associated inflammation, oxidative stress, and tissue degeneration. Targeting the AGE-RAGE pathway has therefore become a central focus in anti-ageing research (Li et al., 2025). The accumulation of AGEs and their interaction with RAGE activate signaling pathways implicated in degenerative disorders such as diabetes, cardiovascular disease, and neurodegeneration (Wang et al., 2024). Inhibiting AGE formation, blocking RAGE activation, or interrupting downstream signaling could mitigate inflammation and tissue injury (Salazar et al., 2021). Importantly, these pathways can be modulated through pharmacological interventions as well as lifestyle factors such as dietary antioxidants, providing a practical framework for promoting healthy ageing (Chen, 2024).

Following the discovery of the pathogenesis associated with the Maillard reaction and the identification of the AGE-RAGE signaling axis, considerable research efforts have been directed toward inhibiting these processes (Al-Abbasy et al., 2024). The early 2000s witnessed the development of synthetic AGE inhibitors such as aminoguanidine and pyridoxamine, as well as RAGE-antagonistic peptides (RAPs) engineered to block AGE-RAGE binding. These synthetic inhibitors often contain functional groups designed to interact with the RAGE binding pocket, mimicking the physicochemical properties of AGEs (Kong et al., 2025). Although these agents show therapeutic promise in mitigating age-related diseases, limitations including low bioavailability, poor specificity, and safety concerns have constrained their clinical utility (Ngcobo, 2025). Consequently, attention has shifted toward natural or biochemical inhibitors, which tend to offer greater tolerability, multi-target activity, and holistic physiological benefits (Láng et al., 2024).

Naturally occurring compounds such as flavonoids, phenolic acids, and alkaloids, commonly found in dietary

sources are biodegradable, better tolerated for long-term use, and possess multiple functional groups (hydroxyl, methoxy, and carbonyl) that enable free radical scavenging and protection against protein and DNA modification (Rodríguez-Negrete et al., 2024). Their antioxidant, anti-inflammatory, and metal-chelating properties further underscore their therapeutic potential (Gulcin, 2025). Therefore, this review aims to synthesize current knowledge on the molecular mechanisms underlying the AGE-RAGE signaling pathway, evaluate existing inhibition strategies, and highlight emerging prospects for phytochemical modulation as potential therapeutic interventions in ageing and degenerative diseases. By consolidating emerging biochemical, molecular, and computational evidence on AGE-RAGE modulation, rational design of next-generation anti-ageing interventions might be engendered. Revelations from this study may guide future studies toward mechanism-driven therapeutic developments, thereby facilitating the identification of safer long-term modulators of age-related signaling as well as support the translation of phytochemical-based strategies into clinically relevant anti-ageing frameworks.

METHODS

Review Design and Reporting Framework

This review was conducted and reported using the guidelines provided by the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (Page et al., 2021). This framework was adopted to ensure transparency, reproducibility, and methodological rigor in the identification, screening, eligibility assessment, and inclusion of relevant literature. The PRISMA flow diagram illustrating the study selection process is provided in Figure 1.

Literature Search Strategy

A comprehensive and systematic literature search was performed across major academic databases, including PubMed, Scopus and the Web of Science as well as supplementary sources like Google Scholar and ResearchGate, to identify studies relevant to glycation chemistry, ageing, age-related diseases, advanced glycation end-products (AGEs), and their receptor (RAGE). Searches were conducted using Boolean operators and combinations of controlled and free-text keywords, including but not limited to: “advanced glycation end-products,” “AGE-RAGE signaling,” “glycation chemistry,” “oxidative stress,” “anti-glycation compounds,” “phytochemicals,” “natural inhibitors,” and “degenerative diseases.”

The search was restricted to peer-reviewed articles published in English between January 2005 to January 2026 to ensure inclusion of contemporary and methodologically sound research. To enhance coverage, manual reference screening of relevant review articles and primary studies was also undertaken.

Study Selection and Screening Process

All retrieved records were imported into the Zotero reference management system (version 7.0.32), where automated and manual duplicate removal was performed prior to title and abstract screening. Study selection followed a two-stage screening process consistent with PRISMA recommendations:

1. Title and abstract screening was conducted to exclude clearly irrelevant studies.
2. Full-text assessment was subsequently performed to evaluate eligibility based on predefined inclusion and exclusion criteria.

Screening and eligibility assessments were conducted independently by the authors, with discrepancies resolved through discussion and consensus. Only studies directly relevant to AGE-RAGE mechanisms and anti-ageing biochemical processes were retained.

Inclusion and Exclusion Criteria

Studies were included if they met one or more of the following:

- Provided mechanistic insight into the chemistry of ageing, AGE formation, or RAGE activation.
- Examined biochemical, molecular, pharmacological, or computational aspects of the AGE-RAGE axis.
- Investigated inhibitory or modulatory strategies targeting AGE formation or AGE-RAGE interaction.
- Reported structural, chemical, or biological properties of phytochemicals with antioxidant, anti-inflammatory, antiglycation, or metal-chelating activity relevant to AGE-RAGE modulation.

Studies were excluded if they were:

- Non-peer-reviewed sources (conference abstracts, editorials, commentaries).
- Not directly related to molecular, biochemical, or mechanistic aspects of the AGE-RAGE pathway.
- Lacking sufficient methodological detail or experimental validation.
- Purely descriptive without mechanistic or analytical relevance.

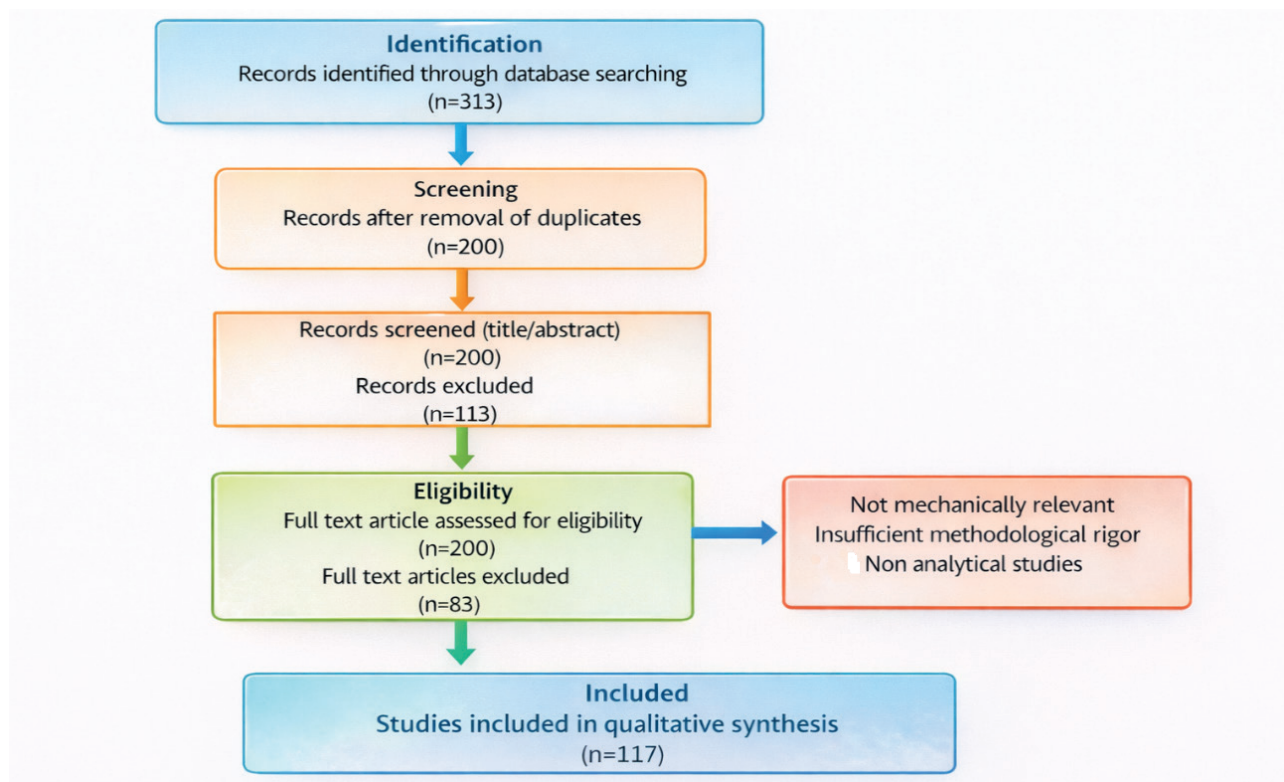


Figure 1: Model of article screening (PRISMA 2020)

Methodological Quality and Risk-of-Bias Assessment

All eligible studies were subjected to a structured qualitative appraisal in order to address methodological quality and potential bias. Experimental studies were evaluated based on criteria adapted from established risk-of-bias assessment tools, including:

- Clarity of experimental design and objectives
- Appropriateness of analytical or biochemical methods
- Adequacy of controls and reproducibility of results
- Consistency between reported data and conclusions

Computational and theoretical studies were assessed for:

- Validity of computational models and parameters
- Transparency and reproducibility of methodological workflows

Studies which failed to meet the minimum quality thresholds by our assessment were excluded during full-text screening. Given the mechanistic and qualitative nature of the review, quantitative risk-of-bias scoring and meta-analysis were not performed, consistent with PRISMA recommendations for non-interventional systematic reviews.

Data Extraction and Synthesis

Following PRISMA-guided screening, 313 records were initially identified. After duplicate removal and title/abstract screening, 200 articles were assessed at the full-text level. Out of these, 117 studies met the inclusion criteria and were retained for qualitative synthesis.

Data extraction focused on:

- Chemical pathways of AGE formation
- Structural and signaling features of RAGE
- Molecular mechanisms of AGE–RAGE inhibition
- Therapeutic and anti-ageing implications of phytochemical modulators

A narrative and thematic synthesis approach was employed to integrate findings across biochemical, pharmacological, and computational domains. Results were organized into thematic sections encompassing AGE chemistry, RAGE structure and signaling, inhibition mechanisms, and emerging phytochemical strategies, enabling identification of mechanistic trends and critical knowledge gaps.

CHEMISTRY OF AGES FORMATION

AGEs constitute a structurally heterogeneous group of biomolecules formed through complex non-enzymatic reactions involving reducing sugars and nucleophilic sites on proteins, lipids, or nucleic acids (Perrone et al., 2020). Their diversity arises from the wide range of precursors and reaction intermediates participating in their synthesis (Sharma et al., 2015). Chemically, AGEs are characterized by the presence of highly reactive carbonyl functional groups, which confer both their chemical reactivity and biological activity, including their propensity to cross-link biomolecules and alter cellular signaling (Nagai et al., 2014). Once formed, AGEs are chemically stable and essentially irreversible, leading to their progressive accumulation within tissues over time (Sadeghi et al., 2023). The formation of AGEs primarily occurs through three fundamental biochemical pathways: the Maillard reaction, the polyol pathway, and lipid peroxidation, each contributing distinct intermediates and structural variants to the overall AGE pool.

The Maillard Pathway

The Maillard reaction is a non-enzymatic chemical process involving a condensation reaction between reducing sugars and amino groups of biomolecules such as proteins, peptides, or amino acids (Twarda-Clapa et al., 2022). Reducing sugars possess highly reactive free aldehyde or ketone groups, which readily react with the nucleophilic amino group of amino acid residues, initiating the early stages of AGE formation (Al-Abbasy et al., 2024). This initial reaction yields an unstable Schiff base intermediate, which subsequently undergoes an Amadori rearrangement, producing more stable intermediates known as Amadori products (El Hosry et al., 2025). The Amadori rearrangement involves an intramolecular shift in bonding that converts the initial imine linkage into a ketoamine structure, thereby stabilizing the intermediate (Smita. Burhade et al., 2025). In glucose- and fructose-mediated reactions, the resulting rearrangements yield glucosamine and fructosamine derivatives, respectively (Takeuchi et al., 2010). The Amadori products formed during this process can undergo a cascade of dehydration, oxidation, and cross-linking reactions, leading to the generation of chemical complex and biologically active AGEs with pathogenic potential (Ho et al., 2024). Once formed, these AGEs are highly stable and irreversible, contributing to their persistence and accumulation in biological systems. Under oxidative conditions, Schiff bases may also bypass the Amadori stage entirely through the Namiki pathway,

directly yielding reactive dicarbonyl compounds that accelerate AGE formation (Thakor et al., 2024).

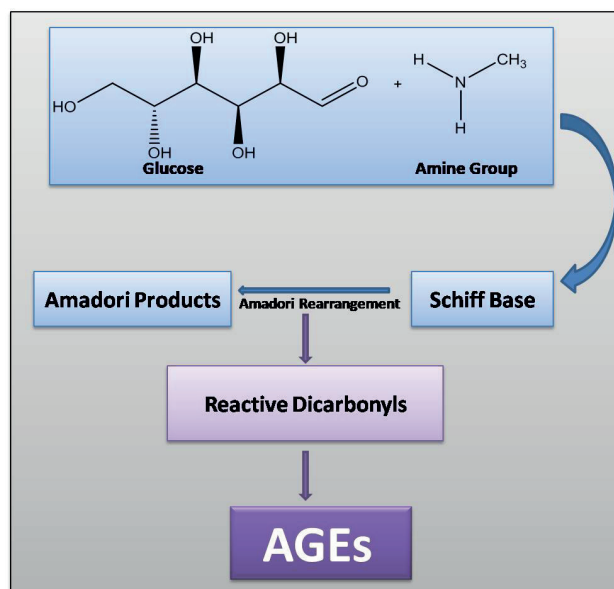


Figure 2: Formation of AGEs from the Maillard Pathway

The Polyol Pathway

The polyol pathway represents an alternative metabolic route for glucose utilization that significantly contributes to the formation of AGEs. In this pathway, glucose or other aldoses are first reduced to sorbitol through the catalytic action of the enzyme aldose reductase, and subsequently oxidized to fructose by sorbitol dehydrogenase (Delannoy et al., 2025). This two-step redox sequence not only alters carbohydrate metabolism but also enhances the generation of reactive carbonyl intermediates that promote glycation. The metabolic conversion of glucose to fructose via the polyol pathway increases AGE formation since fructose is a more potent glycating agent than glucose, reacting with amino residues at a markedly higher rate (Yamaguchi and Nagai, 2025). Furthermore, excessive sorbitol accumulation within tissues can induce osmotic stress, cellular swelling, and secondary oxidative damage, all of which potentiate inflammatory signaling and lipid peroxidation. These conditions create a biochemical environment that amplifies AGE production and accelerates tissue injury (Clarke et al., 2024). Thus, dysregulation of the polyol pathway not only contributes to AGE accumulation but also establishes a mechanistic link between hyperglycemia, oxidative stress, and inflammation, which are key processes underlying ageing and diabetic complications.

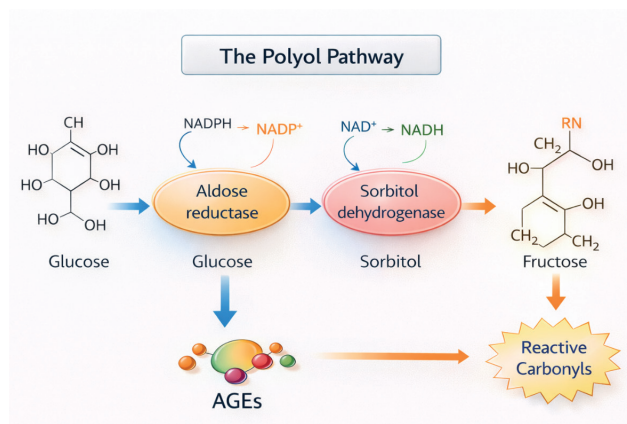


Figure 3: Formation of AGEs from the polyol pathway

The Lipid Peroxidation Pathway

The lipid peroxidation pathway refers to the oxidative degradation of lipids, primarily polyunsaturated fatty acids (PUFAs), resulting from the attack of ROS such as hydroxyl radicals, superoxide anions, and peroxides on cellular membranes (Valgimigli, 2023). This chain reaction leads to the generation of lipid radicals and hydroperoxides, which can propagate further oxidative damage across cell membranes. The process compromises membrane integrity, alters cellular signaling, and disrupts metabolic homeostasis, events that collectively contribute to the biochemical manifestations of ageing and degenerative diseases (Zheng et al., 2024). Although the body possesses endogenous defense mechanisms, including antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase, as well as non-enzymatic antioxidants like vitamin E and coenzyme Q10, prolonged or excessive oxidative stress can overwhelm these protective systems (Pizzino et al., 2017). The resulting accumulation of lipid peroxidation by-products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) induces secondary modifications in proteins and nucleic acids, further amplifying oxidative injury (Yang et al., 2024). Consequently, chronic lipid peroxidation represents a major biochemical pathway linking oxidative stress, AGE formation, and cellular senescence, underscoring its pivotal role in the ageing process and the pathogenesis of various chronic diseases.

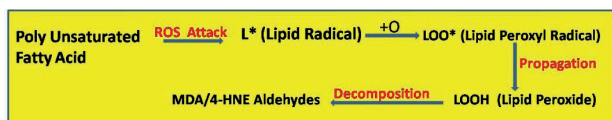


Figure 4: Formation of AGEs from the Lipid Peroxidation Pathway

MAJOR BIOLOGICALLY RELEVANT AGES IN HUMANS

Despite the extensive structural diversity of AGEs described in-vitro, pathological relevance in humans appears to be restricted to a limited subset that forms efficiently in-vivo, accumulates in tissues, and is reproducibly linked to disease. The pathogenic relevance of AGEs is determined less by their chemical diversity than by their ability to accumulate in tissues and engage RAGE-dependent signaling pathways implicated in chronic inflammation and metabolic disease (Ramasamy et al., 2005). This distinction is critical, as AGEs are not biologically interchangeable; their pathological relevance is governed by formation kinetics, chemical stability, resistance to proteolytic turnover, tissue persistence, abundance in long-lived proteins, and their ability to perturb protein structure, mechanical properties, or cellular signaling pathways (Bierhaus and Nawroth, 2009).

In human physiology and pathology, AGEs induce signaling effects through both receptor-independent mechanisms such as protein crosslinking, altered enzymatic activity, and matrix stiffening and receptor-dependent mechanisms, most notably via engagement of the receptor for advanced glycation end products (RAGE) (Ramasamy et al., 2005). The biological significance of AGEs extends beyond mere chemical detectability, encompassing their in-vivo persistence, accumulation in long-lived biomolecules, and capacity to drive functional impairment and pathology (Ramasamy et al., 2012). The AGEs discussed below are classified according to the strength of evidence supporting their accumulation, biological activity, and clinical relevance in humans.

AGEs with Established Clinical and Pathophysiological Relevance

Among the large number of chemically defined AGEs, N ϵ -(carboxymethyl)lysine (CML), N ϵ -(carboxyethyl)lysine (CEL), glucosepane, and pentosidine are the most consistently detected in human tissues and biological fluids. These species are regarded as major drivers of AGE-mediated pathology, preferentially accumulating in long-lived proteins such as collagen, elastin, lens crystallins, and basement membrane components, where limited turnover permits cumulative damage. Their formation is strongly accelerated by chronic hyperglycemia, oxidative stress, inflammation, and defective carbonyl detoxification (Sell et al., 2005). CML and CEL are non-crosslinking AGEs formed

predominantly through glycoxidative and dicarbonyl-driven pathways, respectively. CML arises from oxidative cleavage of early glycation intermediates as well as from reactions involving glyoxal, while CEL is formed primarily through modification of lysine residues by methylglyoxal (Sugawa et al., 2024). Although these AGEs do not directly crosslink proteins, they serve as robust molecular markers of carbonyl stress and are strongly associated with metabolic and degenerative disorders, including diabetes mellitus, cardiovascular disease, chronic kidney disease, and age-related tissue dysfunction. Importantly, both CML and CEL are recognized ligands for RAGE, enabling them to couple protein glycation to downstream pro-inflammatory, pro-oxidative, and pro-fibrotic signaling cascades (Khan et al., 2024).

In contrast, glucosepane and pentosidine are bifunctional crosslinking AGEs that exert profound effects on tissue biomechanics and structural integrity (Bronowicka-Szydełko et al., 2024). Glucosepane, the most abundant protein crosslink identified in human collagen, forms slowly through complex multistep reactions involving Amadori intermediates and reactive dicarbonyl species. Its exceptional chemical stability and resistance to enzymatic degradation lead to progressive accumulation with age, contributing to extracellular matrix stiffening, reduced vascular compliance, impaired cell-matrix interactions, and compromised tissue repair (Sell et al., 2005). Pentosidine, although less abundant than glucosepane, serves as a well-established fluorescent marker of cumulative glycation and oxidative stress and is frequently elevated in diabetic complications, renal failure, and inflammatory conditions (deRamon et al., 2022).

AGEs with Emerging or Context-Dependent Biological Relevance

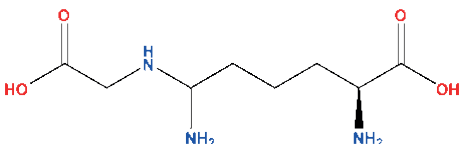
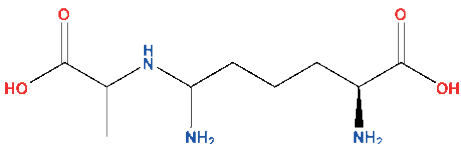
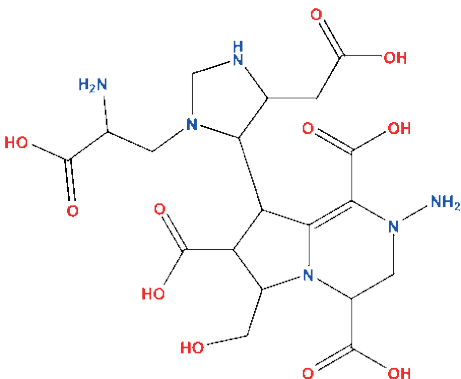
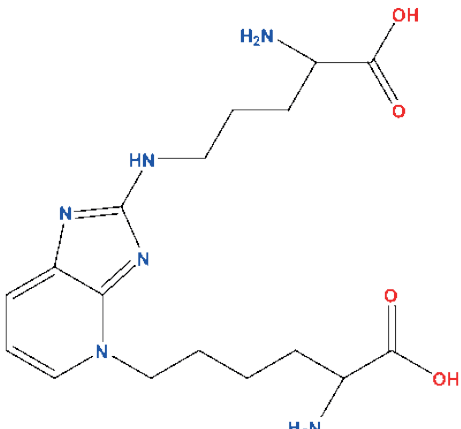
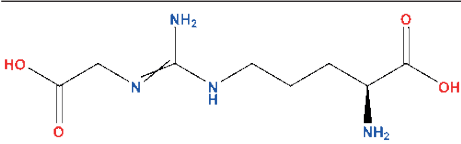
Several additional glycation products have been detected in human tissues and disease states beyond the dominant AGEs described above. These include N ϵ -(carboxymethyl)arginine (CMA), argpyrimidine, crossline, and vesperlysine. Many of these species arise preferentially under conditions of intense oxidative stress or elevated dicarbonyl flux and are often regarded as indicators of advanced glycoxidative damage rather than primary drivers of pathology (Ho et al., 2024; Lin et al., 2012). Argpyrimidine is generated through methylglyoxal-mediated modification of arginine residues and has been identified in diabetic tissues and sites of inflammation (Gomes et al., 2005). Although it can induce conformational alterations in

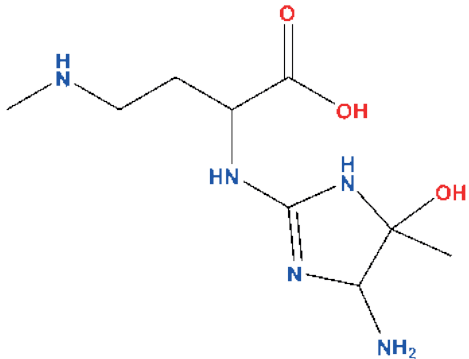
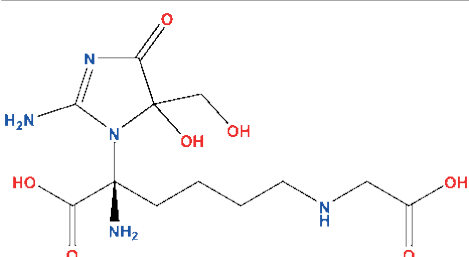
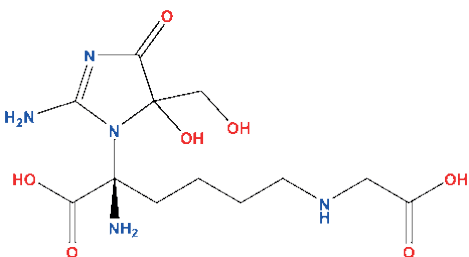
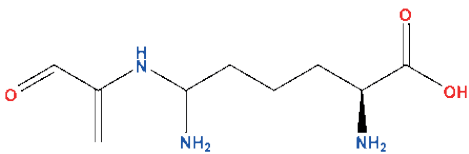
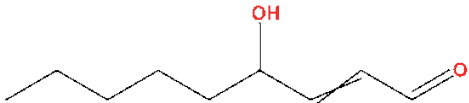
proteins and, under certain conditions, engage RAGE-dependent signaling, its comparatively low abundance suggests a limited contribution relative to dominant AGEs such as CML or glucosepane (Sadowska-Bartosz and Bartosz, 2015). Likewise, crossline and vesperlysine constitute structurally elaborate crosslinking AGEs that, despite their chemical interest, occur at low levels in human tissues. Current evidence suggests that these AGEs may modulate disease processes in a context-dependent manner, potentially amplifying oxidative stress or inflammatory signaling under extreme metabolic dysregulation. However, their contribution to tissue dysfunction appears secondary to that of the dominant AGEs, and their precise roles remain incompletely defined. As such, they are best viewed as complementary markers and modulators of advanced glycation rather than central pathogenic entities (Santiago-Fernández et al., 2025).

Lipoxidation-Derived AGEs and Related Carbonyl Adducts

Reactive aldehyde-derived protein adducts, including malondialdehyde-lysine (MDA-Lys) and 4-hydroxynonenal (4-HNE) conjugates, are often discussed alongside AGEs because of their overlapping biological effects and shared roles in oxidative stress-associated pathologies. However, these adducts are generated primarily through lipid peroxidation rather than carbohydrate-mediated glycation and are therefore more appropriately classified as advanced lipoxidation end products (ALEs) (Yammine et al., 2024). ALEs contribute significantly to protein dysfunction, membrane damage, inflammation, and cellular stress responses and may interact with AGE-responsive receptors. Their formation chemistry, structural features, and biological behavior are distinct from classical sugar-derived AGEs. Conflating AGEs and ALEs risks obscuring mechanistic clarity, particularly in studies focused on carbonyl stress pathways and receptor-mediated signaling (Moldogazieva et al., 2019). Accordingly, ALEs should be regarded as parallel mediators of oxidative pathology that often coexist with AGEs in chronic disease states, rather than as core members of the AGE family in a strict biochemical sense.

Table 1: Major biologically relevant AGEs

AGE	Precursor compound(s)	Amino acids involved	Biological significance
 Nε-(Carboxymethyl)lysine (CML)	Glyoxal, Ascorbate oxidation products	Lysine	Most abundant AGE in human tissues; biomarker of oxidative stress, ageing, and diabetic complications; formed via glycoxidation and lipid peroxidation.
 Nε-(Carboxyethyl)lysine (CEL)	Methylglyoxal	Lysine	Non-crosslinking AGE; indicates methylglyoxal stress; associated with diabetes, atherosclerosis, and metabolic disorders.
 Glucosepane	3-Deoxyglucosone	Lysine + Arginine	Major protein crosslink in human collagen; accumulates in aged and diabetic tissues; increases tissue stiffness and reduces elasticity.
 Pentosidine	Ribose or Pentose sugars	Lysine + Arginine	Fluorescent crosslink AGE; biomarker for diabetic and renal complications; contributes to vascular stiffness and oxidative stress.
 Nε-(Carboxymethyl)arginine (CMA)	Glyoxylic acid	Arginine	Modifies the guanidino group of arginine; contributes to protein dysfunction and inflammatory signaling in oxidative stress conditions.

AGE	Precursor compound(s)	Amino acids involved	Biological significance
 Argpyrimidine	Methylglyoxal	Arginine	Fluorescent AGE modifying guanidino group; found in neural and vascular tissues; contributes to endothelial dysfunction and neurodegeneration.
 Crossline	Glucose	Lysine + Lysine	Fluorescent protein crosslink; promotes structural rigidity in long-lived proteins; associated with ageing and cataract formation.
 Vesperlysine A (VL-A)	Glucose or 3-deoxyglucosone	Lysine + Arginine	Fluorescent AGE isolated from aged skin collagen; contributes to tissue yellowing and oxidative damage.
 Malondialdehyde-lysine (MDA-Lys)	Malondialdehyde	Lysine	Lipid peroxidation product; forms adducts with lysine residues; marker of oxidative stress, atherosclerosis, and inflammation.
 4-hydroxynonenal (4-HNE)	PUFA oxidation	Cysteine, Lysine, Histidine	Highly reactive lipid-derived aldehyde; forms adducts with proteins; cytotoxic and pro-inflammatory; involved in neurodegeneration and chronic diseases.

RECEPTORS FOR ADVANCED GLYCATION END PRODUCTS (RAGE)

RAGE is a multifunctional cell-surface receptor belonging to the immunoglobulin superfamily, characterized by its ability to recognize and bind a wide range of structurally diverse ligands, including AGEs, S100 proteins, HMGB1, amyloid- β , and pathogen-associated molecules (Cross et al., 2024). This broad ligand recognition capacity gives RAGE a dual functional identity in both physiological and pathological contexts (Radziszewski et al., 2024). Under normal or acute conditions, ligand-induced RAGE activation supports host defense mechanisms, promotes cell survival, and facilitates tissue repair by triggering stress-response and immunomodulatory signaling pathways (Deepu et al., 2024). In contrast, chronic or dysregulated activation of RAGE sustains a persistent cycle of oxidative stress, inflammation, and metabolic imbalance, thereby contributing to the progression of metabolic disorders, vascular dysfunction, neurodegeneration, diabetes and cancer (Chandimali et al., 2025; Giridharan et al., 2021; Ramasamy et al., 2022).

RAGE exists primarily in two structural forms: the membrane-bound RAGE (mRAGE) and the soluble RAGE (sRAGE) (Adamu et al., 2024). mRAGE is the signaling-competent form responsible for mediating intracellular responses that promote cell survival, immune activation, and maintenance of tissue homeostasis during infection or injury (Watanabe and Son, 2021). Structurally, mRAGE comprises three extracellular immunoglobulin-like domains- the variable (V) domain and two constant domains (C1 and C2), followed by a single transmembrane helix and a short cytoplasmic tail essential for intracellular signal transduction (Radziszewski et al., 2024). Ligand recognition occurs predominantly in the V-domain, which contains conserved basic amino acid residues such as arginine and lysine that generate an electropositive surface, enabling strong interactions with negatively charged ligands (Ijaz et al., 2023; Olufunmilayo, et al., 2023). In contrast, sRAGE functions as a natural decoy receptor. Lacking the transmembrane and cytoplasmic domains, it consists solely of the three extracellular domains (V, C1, and C2). sRAGE binds circulating RAGE ligands, thereby preventing their interaction with mRAGE and attenuating excessive inflammation and oxidative signaling (Yue et al., 2022). It is generated through either alternative mRNA splicing or proteolytic shedding of the extracellular domain of mRAGE (Ho et al., 2024).

RAGE expression varies by cell type and developmental stage. It is highly expressed during embryogen-

esis, where it contributes to growth and differentiation, but its expression decreases significantly in adult tissues. Elevated RAGE levels in adults typically reflect pathological conditions characterized by heightened oxidative or inflammatory stress.

AGE-RAGE SIGNALING PATHWAY

The AGE-RAGE signaling pathway is initiated when AGEs interact with the RAGE, a process governed by a combination of electrostatic, hydrophobic, hydrogen-bonding, and aromatic interactions (Gutowska et al., 2023). RAGE's structural flexibility and the presence of multiple ligand-binding surfaces across its extracellular domains enable it to recognize chemically diverse ligands (Comajuncosa-Creus et al., 2024). The V-domain, in particular, provides specificity through its arrangement of basic residues while still accommodating a broad range of negatively charged or aromatic ligands (Dascalu et al., 2024). AGE-RAGE binding occurs mainly through three interaction types: (i) ionic interactions between the electropositive residues of RAGE and electronegative AGE functional groups such as carboxylates, sulfates, and phosphates; (ii) hydrogen bonding between amine or hydroxyl groups on RAGE and carbonyl groups on AGEs; and (iii) π - π and cation- π interactions involving aromatic amino acid side chains and the ring structures of AGE molecules (Stevenson et al., 2023). As AGEs accumulate with aging, particularly in long-lived macromolecules such as collagen, these carbonyl-rich structures increasingly engage RAGE and initiate downstream signaling (Wang et al., 2024). Central to this pathway are the activation of NADPH oxidase and the amplification of protein glycooxidation.

Upregulation of NADPH Oxidase

NADPH oxidase (NOX) is a membrane-associated enzyme complex responsible for controlled production of reactive oxygen species (ROS) in physiological signaling (Schröder, 2024). AGE-RAGE engagement activates NOX, triggering electron transfer from NADPH to molecular oxygen. Electrons released from the nicotinamide ring of NADPH as a hydride ion (H^-) are relayed through the NOX complex to oxygen, producing superoxide radicals ($O_2^{\bullet-}$) and subsequently other ROS such as hydroxyl radicals ($\bullet OH$) (Liu et al., 2025; Bastos et al., 2025). Iron ions in the labile iron pool further amplify ROS production via Fenton chemistry (Jomova et al., 2023). The resulting oxidative burst drives lipid peroxidation, protein oxidation, and DNA damage. Lipid per-

oxides weaken membrane integrity and decompose into toxic aldehydes including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which further propagate oxidative injury (Zhao et al., 2023). Oxidized proteins accumulate disulfides and sulfonic acids, leading to misfolding, aggregation, and loss of enzymatic function. DNA oxidation, particularly the formation of 8-oxo-2'-deoxyguanosine, promotes mutagenic mispairing, mitochondrial dysfunction, and impaired genomic repair which are hallmarks of aging and degenerative pathology (Zhao et al., 2023).

Protein Glycooxidation and Cross-Linking

AGE-RAGE activation also accelerates protein glycooxidation, promoting the formation of irreversible crosslinks in long-lived extracellular matrix proteins such as collagen and elastin. Major AGE-derived crosslinks including pentosidine, methylglyoxal-derived hydroimidazolone (MG-H1), and glucosepane, accumulate progressively and stiffen tissue structures (Meiliana et al., 2024). Binding of AGEs to RAGE induces conformational changes that trigger a redox-sensitive intracellular cascade (Nair et al., 2019). Elevated ROS levels enhance the formation of new AGEs, establishing a self-amplifying loop in which oxidative stress increases glycation, and glycation increases oxidative stress (Peng et al., 2024). Over time, this feedback cycle leads to persistent RAGE activation, extensive crosslinking of extracellular matrix proteins, reduced molecular mobility, and disruption of normal receptor-ligand dynamics. These processes contribute to the structural deterioration and functional decline characteristic of aging tissues (Benigni et al., 2024).

MECHANISM OF AGE-RAGE AXIS INHIBITION

Inhibition of the AGE-RAGE axis focuses on two complementary strategies: reducing the endogenous formation of AGEs and preventing the interaction of existing AGEs with the RAGE (Semchyshyn, 2024). The central goal is to either neutralize reactive glycation intermediates before they generate AGEs or to sequester AGEs in forms that render them incapable of binding to RAGE. Interventions targeting AGE formation generally act through carbonyl trapping, free radical scavenging, and metal chelation, three mechanistic pathways that collectively limit glycooxidative stress in biological systems (Yadav et al., 2023). From an anti-ageing perspective, these strategies tackle a key upstream knowledge gap by showing that AGE accumulation is not an una-

voidable outcome of ageing, but a process amenable to chemical modulation. Elucidating the molecular mechanisms by which AGE formation can be suppressed establishes a rational basis for interventions aimed at decelerating the underlying biochemical drivers of ageing, rather than simply managing its downstream manifestations.

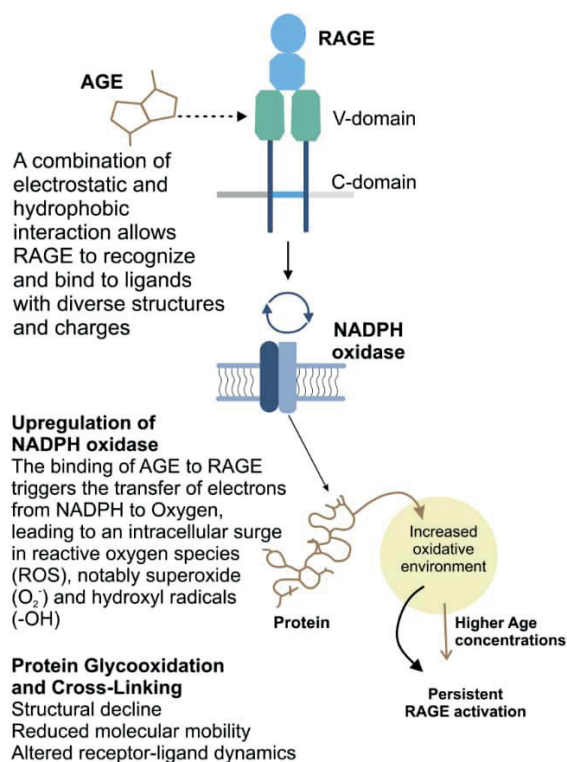


Figure 5: The AGE-RAGE axis describes the interaction between accumulated advanced glycation end products and their cellular receptor, RAGE, triggering oxidative stress and pro-inflammatory signaling. This interaction creates a self-amplifying loop that promotes chronic inflammation, vascular dysfunction, and tissue degeneration

Carbonyl Trapping

Carbonyl trapping is a frontline defense against AGE formation because it directly intercepts the highly reactive carbonyl electrophiles particularly dicarbonyl compounds and methylglyoxal that drive early Maillard chemistry. These carbonyl species readily attack nucleophilic amino acid residues such as lysine and arginine, initiating irreversible AGE formation. Carbonyl trapping agents contain nucleophilic functional groups that rapidly react with these dicarbonyl intermediates to form chemically stable, non-toxic adducts, effectively neutral-

izing the glycation potential of the intermediates (Uceda et al., 2024b). By preventing carbonyl–protein interactions, this mechanism interrupts the Maillard reaction at its early stages, maintaining the integrity of free amino groups and substantially reducing downstream AGE accumulation (Hidalgo and Zamora, 2023). This mechanistic insight addresses a critical research gap by demonstrating that early chemical interception of glycation intermediates can preserve protein integrity and retard the progressive structural deterioration of long-lived biomolecules that drive tissue ageing.

Free Radical Scavenging

Oxidative stress accelerates glycoxidation reactions and amplifies AGE production. Antioxidants counteract this process by donating electrons or hydrogen atoms to neutralize reactive oxygen species (ROS), thereby suppressing oxidative pathways that promote AGE formation. Polyphenols, particularly those enriched with hydroxyl-substituted aromatic rings, serve as highly effective free radical scavengers due to their ability to stabilize unpaired electrons and terminate radical propagation chains (Andrés et al., 2023). By reducing ROS levels, antioxidants lower glycoxidative pressure on proteins and lipids, creating a biochemical environment less conducive to AGE generation and RAGE activation. Importantly, this reinforces the concept that oxidative stress is not only a parallel ageing pathway but a catalytic accelerator of AGE–RAGE signaling, thereby positioning antioxidant modulation as a strategic lever in slowing ageing-related molecular damage.

Metal Chelation

Transition metals such as Fe^{2+} and Cu^{2+} catalyze oxidative steps in the Maillard reaction through Fenton-type reactions that generate hydroxyl radicals. Metal chelators inhibit AGE formation by binding these catalytic metal ions through electron-donating atoms especially oxygen or nitrogen, forming stable, inert complexes that prevent metal-mediated oxidation (Ohiagu et al., 2025). Compounds with ortho-dihydroxy ($-\text{OH}$) functional groups or phenolic structures bearing carboxylic acid substituents exhibit strong chelating capacity and effectively suppress metal-induced glycation (Lee et al., 2023). This mechanism complements antioxidant and carbonyl-trapping pathways by targeting a different but critical driver of glycoxidative stress.

MODULATING THE AGE–RAGE SIGNALING

Blocking the AGE–RAGE axis focuses on preventing the physical interaction between AGEs and their cellular receptor, RAGE, thereby suppressing downstream oxidative and inflammatory signaling. This strategy primarily involves the use of molecules that competitively occupy the ligand-binding pocket of RAGE, mimicking structural or chemical features of AGE ligands. Binding occurs through a combination of hydrogen bonding, ionic interactions, van der Waals forces, and hydrophobic effects that stabilize inhibitor–receptor interactions within the RAGE V-domain (Khateri et al., 2024). Among promising inhibitors, sulfonated flavonoid derivatives such as sulfoquercetin demonstrate significantly enhanced affinity for RAGE. Their negatively charged sulfonate and carboxylate groups form strong ionic interactions with conserved cationic residues (e.g., Arg and Lys) within the electropositive binding surface of the V-domain. These electrostatic interactions (Table 2) are complemented by the ability of flavonoid aromatic rings to engage in π – π stacking and cation– π interactions with the aromatic amino acids in RAGE, further stabilizing the ligand–receptor complex (Arellanes-Lozada et al., 2023).

In addition to ionic and aromatic interactions, many synthetic inhibitors are specifically designed to fit into the non-polar hydrophobic pockets of the receptor. Occupation of these pockets enhances binding affinity by reducing solvent exposure and maximizing van der Waals contacts. Because the V-domain possesses several hydrophobic and aromatic residues, these inhibitors can effectively outcompete endogenous AGEs for binding, thereby preventing receptor activation. Another important strategy for modulating AGE–RAGE signaling is the use of soluble RAGE (sRAGE) as a circulating decoy receptor. Although sRAGE does not inhibit the binding of AGEs to membrane-bound RAGE (mRAGE) directly, it reduces AGE–RAGE signaling by sequestering AGEs in the extracellular environment (Reddy et al., 2023). Unlike mRAGE, sRAGE lacks the transmembrane segment and the intracellular cytoplasmic tail required for signal transduction, meaning that AGE binding to sRAGE does not initiate downstream inflammatory or oxidative pathways (Rojas et al., 2024). Therefore, elevated levels of sRAGE serve a protective role by limiting the availability of AGEs for interaction with mRAGE. This underscores the fact that ageing-related inflammation and oxidative stress are not passive consequences of molecular damage but are actively sustained by ligand–receptor signaling, which is amenable to pharmacological modulation.

Table 2: Reactive Groups Involved in AGE–RAGE Complex Formation

RAGE (V-domain)	Functional Groups	Properties
Arginine	Guanidinium group	Strongly basic, cationic at pH 7.4
Lysine	ε-Amino group	Basic, cationic at pH 7.4
Serine, Threonine	–OH groups	H-bond donors/acceptors
Tyrosine, Phe	Aromatic rings	π–π stacking sites
AGEs	Functional Groups	Properties
Carboxymethyllysine	–COOH	Negatively charged
Pyrraline, MG-H1	Imidazole, carbonyls	H-bonding, polar
Crosslinked AGEs	Dicarbonyl bridges	Reactive, electrophilic

PHYTOCHEMICAL MODULATION OF THE AGE–RAGE PATHWAY

The interaction between AGEs and their cognate receptor (RAGE) constitutes a pivotal molecular nexus that amplifies oxidative stress, inflammation, and cumulative tissue injury across numerous metabolic, vascular, and age-associated pathologies. Engagement of RAGE by structurally diverse AGEs triggers a cascade of intracellular events including ROS overproduction, NF-κB activation, cytokine release, and sustained feedback upregulation of RAGE itself that perpetuate a chronic pro-oxidant and pro-inflammatory milieu. This self-reinforcing loop is now recognized as a central driver of diabetic complications, neurodegeneration, renal fibrosis, endothelial dysfunction, and accelerated biological ageing. Despite growing recognition of this axis, a critical gap remains in translating chemical insights into safe, long-term anti-ageing interventions capable of modulating AGE–RAGE signaling without adverse effects.

Amid growing global interest in safer, naturally derived therapeutic candidates, phytochemicals especially polyphenols, alkaloids, and tannins have gained prominence for their capacity to modulate key biochemical determinants of glycoxidative stress (Montserrat Corona-España et al., 2024). These plant-derived metabolites operate through multilayered and complementary mechanisms, making them uniquely suited for targeting the complex molecular architecture of the AGE–RAGE axis.

Polyphenols

Chemically, polyphenols are defined by aromatic ring systems bearing multiple hydroxyl (–OH) substituents, a structural arrangement that confers pronounced redox activity through facile electron and hydrogen atom donation. Representative compounds such as quercetin, catechin, and resveratrol possess conjugated π-electron systems and phenolic hydroxyl groups making them able to stabilize radical intermediates, thus underpinning their widely reported antioxidant effects. In in-vitro chemical and cell-free assays, these polyphenols efficiently scavenge reactive oxygen species and inhibit lipid peroxidation at low micromolar concentrations (typically 1–50 μM), while ortho- and para-dihydroxyl motifs enable chelation of redox-active transition metals (e.g., Fe²⁺, Cu²⁺), limiting Fenton-type radical generation (Láng et al., 2024).

In cellular models, polyphenols demonstrate additional redox-dependent modulation of signaling pathways, though these effects are concentration-sensitive and context-dependent. Quercetin and catechin have been reported to suppress activation of redox-sensitive transcription factors such as NF-κB and AP-1 in inflammatory cell lines, typically at concentrations ≥10 μM, while resveratrol modulates AMPK- and sirtuin-associated pathways involved in metabolic stress responses (Lee et al., 2023; Ravindranath and Ravindranath, 2011; Wu et al., 2011). Importantly, these cellular responses are frequently accompanied by demonstrable decreases in intracellular oxidative stress, indicating that the observed modulation of signaling pathways is largely a downstream consequence of antioxidant action rather than the result of specific high-affinity receptor–ligand interactions (Terao, 2023). Evidence from in-vivo animal models supports a broader biological impact of polyphenols but also highlights pharmacokinetic constraints. Oral administration of quercetin, catechin, or resveratrol in rodents (commonly 10–100 mg/kg/day) reduces systemic markers of oxidative stress, inflammation, and microbial burden in disease models characterized by redox imbalance (Alharbi et al., 2025; Navarro-Cruz et al., 2024). These effects likely arise from the combined contribution of the parent compounds and their circulating metabolites, some of which may preserve residual antioxidant or metal-chelating activity. However, extensive biotransformation, rapid systemic elimination, and restricted tissue distribution substantially hinder straightforward translation of animal dosing outcomes to human therapeutic settings. Human data remain comparatively limited and largely indirect. While epidemiological and short-term intervention studies asso-

ciate polyphenol-rich diets with reduced oxidative and inflammatory biomarkers, plasma concentrations of unmetabolized polyphenols typically remain in the low nanomolar to submicromolar range. At these levels, direct radical scavenging is unlikely to be the dominant mechanism, implying that long-term modulation of redox homeostasis may arise from cumulative, metabolite-driven, or adaptive biological responses rather than acute antioxidant chemistry.

Alkaloids

Alkaloids characterized by the presence of nitrogen-containing heterocyclic rings, a feature that imparts basicity, electronic density, and a strong propensity for interaction with biological macromolecules. This chemical architecture distinguishes alkaloids from polyphenols and underlies their characteristic high-affinity binding to enzymes, nucleic acids, and neurotransmitter receptors. Representative compounds such as berberine, morphine, and caffeine illustrate the functional diversity of this class, spanning planar aromatic cations, rigid polycyclic scaffolds, and methylxanthine systems. These structural features enable alkaloids to engage biological targets primarily through π - π stacking, hydrogen bonding, electrostatic interactions, and intercalation-like behavior, rather than through direct redox chemistry (Srivastava and Singh, 2020). In *in-vitro* systems, alkaloids act as potent enzyme and receptor modulators, often at low micromolar or submicromolar concentrations. Berberine inhibits a range of enzymes involved in glucose metabolism, oxidative stress regulation, and inflammatory signaling, while also demonstrating affinity for DNA through groove binding and partial intercalation, leading to altered transcriptional activity (Zych et al., 2020). Caffeine, in contrast, exerts weaker direct enzyme inhibition but efficiently antagonizes adenosine receptors and modulates phosphodiesterase activity at higher micromolar concentrations (Pohanka and Dobes, 2013). Morphine shows limited enzyme inhibition but exhibits strong, selective binding to opioid receptors, highlighting that alkaloid bioactivity is dictated more by target specificity than by shared chemical reactivity. Antimicrobial effects observed in cell-free and microbial assays are frequently linked to enzyme inhibition or disruption of nucleic acid function rather than nonspecific cytotoxicity.

In cellular models, alkaloids predominantly exert indirect biological effects mediated by enzyme inhibition and receptor engagement, rather than direct scavenging of reactive species (García-Muñoz et al., 2024). Berberine suppresses inflammatory and metabolic signaling cascades by inhibiting kinases and transcriptional

regulators downstream of enzyme-controlled pathways, while caffeine alters intracellular signaling by modulating cyclic nucleotide turnover and neurotransmitter receptor activity. These effects are typically observed at concentrations ranging from 5–50 μ M, although sensitivity varies widely depending on cell type and target expression. Importantly, alkaloids often influence multiple signaling nodes simultaneously, reflecting their ability to interact with conserved structural motifs across different proteins (Jiang et al., 2024). Evidence from *in-vivo* studies confirm that alkaloids function as systemic modulators of metabolic and neural signaling with attendant pharmacokinetic constraints. Berberine administration in rodents (commonly 50–150 mg/kg/day) improves metabolic parameters, reduces inflammatory mediators, and indirectly attenuates oxidative and glycoxidative stress, effects attributed to enzyme inhibition and altered signaling rather than direct antioxidant activity (Ai et al., 2021). Caffeine, at physiologically relevant doses, exerts well-characterized neurostimulatory and metabolic effects through central and peripheral receptor modulation (Song et al., 2024b). Human data reinforce the view that alkaloids are pharmacologically potent but mechanistically selective agents.

Tannins

Tannins represent a structurally distinct subgroup of polyphenols defined by highly polymeric phenolic frameworks bearing dense arrays of hydroxyl (–OH) groups, thus making them excellent candidates for metal ion coordination and macromolecular binding. Representative compounds include tannic acid, gallotannins, and ellagitannins, whose multiple galloyl or hexahydroxydiphenoyl units generate multidentate binding sites unavailable to low-molecular-weight phenolics. This chemical architecture underlies the characteristic astringency of tannins and distinguishes their mode of action from monomeric antioxidants (Cosme et al., 2025). In *in-vitro* chemical and cell-free systems, tannins exhibit the strongest metal-chelating and protein-binding capacity among plant phenolics, forming stable complexes with transition metals such as Fe^{2+} and Cu^{2+} at low micromolar concentrations. Through this mechanism, tannins effectively suppress metal-catalyzed redox cycling and hydroxyl radical generation, providing potent protection against oxidative damage. Concurrently, their ability to precipitate proteins through hydrogen bonding and hydrophobic interactions disrupts enzyme activity and structural protein function. These effects are robust and reproducible in simplified systems and are directly attributable to tannin polymer size and hydroxyl density rather than to specific receptor interactions (Karamać, 2009).

In cellular and microbial models, tannins demonstrate broad antioxidant, antimicrobial, and anti-inflammatory activity, though these effects are largely indirect and context-dependent. Antioxidant activity in cells is primarily linked to extracellular or membrane-proximal metal sequestration rather than intracellular radical scavenging, as the large molecular size of tannins limits passive cellular uptake (Camarda et al., 2026). Antimicrobial effects are consistently observed *in-vitro*, and are mediated through protein complexation at the microbial cell surface, enzyme inhibition, and disruption of membrane-associated processes. However, these same protein-binding properties can reduce selectivity and complicate mechanistic interpretation in complex biological systems. Studies on animals support a protective role of tannins against oxidative and microbial damage, particularly in gastrointestinal and inflammatory models. Dietary tannins and tannin-rich extracts reduce oxidative stress markers, inflammatory cytokines, and microbial burden in rodents, effects often attributed to local actions within the gut lumen and mucosal interface (Huang et al., 2018). Importantly, intact tannins show limited systemic bioavailability due to poor absorption and extensive interaction with dietary proteins and gut microbiota. As a result, biological effects observed *in-vivo* are increasingly understood to involve microbial metabolites (e.g., urolithins from ellagitannins) rather than the parent polymers (Cosme et al., 2025). Accordingly, while tannins display strong mechanistic potential as modulators of glycoxidative stress, direct extrapolation to human AGE-RAGE inhibition remains unclear. Their contribution is more plausibly interpreted as indirect modulation of oxidative and inflammatory tone rather than as direct systemic AGE inhibitors, further highlighting the need for metabolite-focused and tissue-level investigations.

Table 3: Bioactive attributes of polyphenols, alkaloids, and tannins in oxidative/glycoxidative stress

Feature	Polyphenols	Alkaloids	Tannins
Basic Structure	Aromatic rings with multiple hydroxyl (-OH) groups	Nitrogen-containing heterocyclic rings	Polymeric phenolic structures with multiple hydroxyl groups
Representative Compounds	Quercetin, Catechin, Resveratrol	Berberine, Morphine, Caffeine	Tannic acid, Gallotannins, Ellagitannins
Major Mechanism of Action	Electron/hydrogen donation; free radical scavenging; metal chelation	Enzyme and receptor binding; interference with DNA and proteins	Metal ion complexation; protein precipitation; ROS suppression

Feature	Polyphenols	Alkaloids	Tannins
Primary Biological Activities	Antioxidant, anti-inflammatory, antimicrobial	Neuroactive, antimicrobial, enzyme inhibitory	Antioxidant, antimicrobial, anti-inflammatory, anticarcinogenic
Dominant Molecular Target	ROS/RNS; transition metals	Enzymes, DNA, neurotransmitter receptors	Transition metals (Fe ²⁺ , Cu ²⁺); microbial proteins
Mode of Antioxidant Defense	Radical scavenging and redox cycling	Indirect modulation via enzyme inhibition	Metal sequestration and protein complexation
Comparative Strength	Strong direct antioxidant and redox modulator	Potent enzyme/receptor modulator	Strongest chelating and protein-binding capacity
Biological Niche	Protection against oxidative stress and inflammation	Regulation of metabolic and neural signaling	Prevention of oxidative and microbial damage

MECHANISTIC GAPS IN AGE-RAGE INTERACTIONS AND COMPUTATIONAL PERSPECTIVES IN DRUG DISCOVERY

Although diverse mechanisms have been elucidated regarding the indirect modulation of AGE toxicity, no phytochemical has yet been conclusively demonstrated to directly block the interaction between AGEs and RAGE. Existing evidence suggests that these compounds primarily modulate upstream processes (e.g., inhibition of AGE formation, carbonyl scavenging) or downstream signaling pathways (e.g., suppression of ROS generation and inflammatory cytokines) rather than interacting at the receptor binding interface itself (Ceramella et al., 2024). This represents a notable gap in our pharmacological understanding, underscoring the need for targeted receptor-level investigations. Current computational approaches can provide a powerful strategy for exploring the potential of phytochemicals as direct RAGE antagonists. In-silico tools such as molecular docking, molecular dynamics (MD) simulations, and pharmacophore modeling can offer detailed insights into ligand-receptor interactions at the atomic scale. Docking studies focusing on the VC1 domain of RAGE, the principal region involved in AGE recognition can reveal, binding affinities of phytochemicals, hydrogen bonding patterns, π - π and cation- π interactions with aromatic and basic residues, as well as structural compatibility and stability within the RAGE binding pocket. Such computational explorations have been implemented to assess phytochemicals and synthetic ligands (Ouamnina et al.,

2024; (Ouamnina et al., 2024; Ikezu et al., 2025; Nwofor et al., 2025). Incorporating MD simulations further enables the evaluation of complex stability, ligand-induced conformational changes, and solvent dynamics over time.

For computational predictions to achieve biological relevance, they must be validated through complementary experimental assays such as fluorescence quenching to measure RAGE–ligand interactions, surface plasmon resonance (SPR) for real-time affinity quantification, and isothermal titration calorimetry (ITC) to determine thermodynamic parameters. Such integrated validation would substantially advance the search for natural AGE–RAGE antagonists.

CONCLUSION

A growing body of evidence underscores the AGE–RAGE axis as a central biochemical driver of oxidative stress, chronic inflammation, and degenerative pathology across multiple organ systems. Its involvement in metabolic dysfunction, vascular remodeling, neurodegeneration, and tissue ageing highlights the pathway as a critical, modifiable node in disease progression. Despite significant advances in elucidating AGE formation routes, RAGE structural biology, and downstream redox-sensitive signaling, substantial mechanistic gaps remain particularly regarding ligand specificity, receptor dynamics, and the interplay between glycooxidation, lipid peroxidation, and cellular stress responses. Addressing these gaps will require integrated chemical, biochemical, and computational approaches capable of resolving molecular events with higher precision. Continued exploration of both endogenous defense mechanisms and exogenous modulators will be essential for developing safe, effective therapeutic strategies that interrupt AGE accumulation, dampen RAGE-mediated signaling, and ultimately mitigate the molecular processes that underpin ageing and degenerative disease.

Ethical approval

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Consent to participate

Not applicable.

Consent to publish

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Chidi Edbert Duru: Conceptualization, Methodology, Data curation, Review.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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