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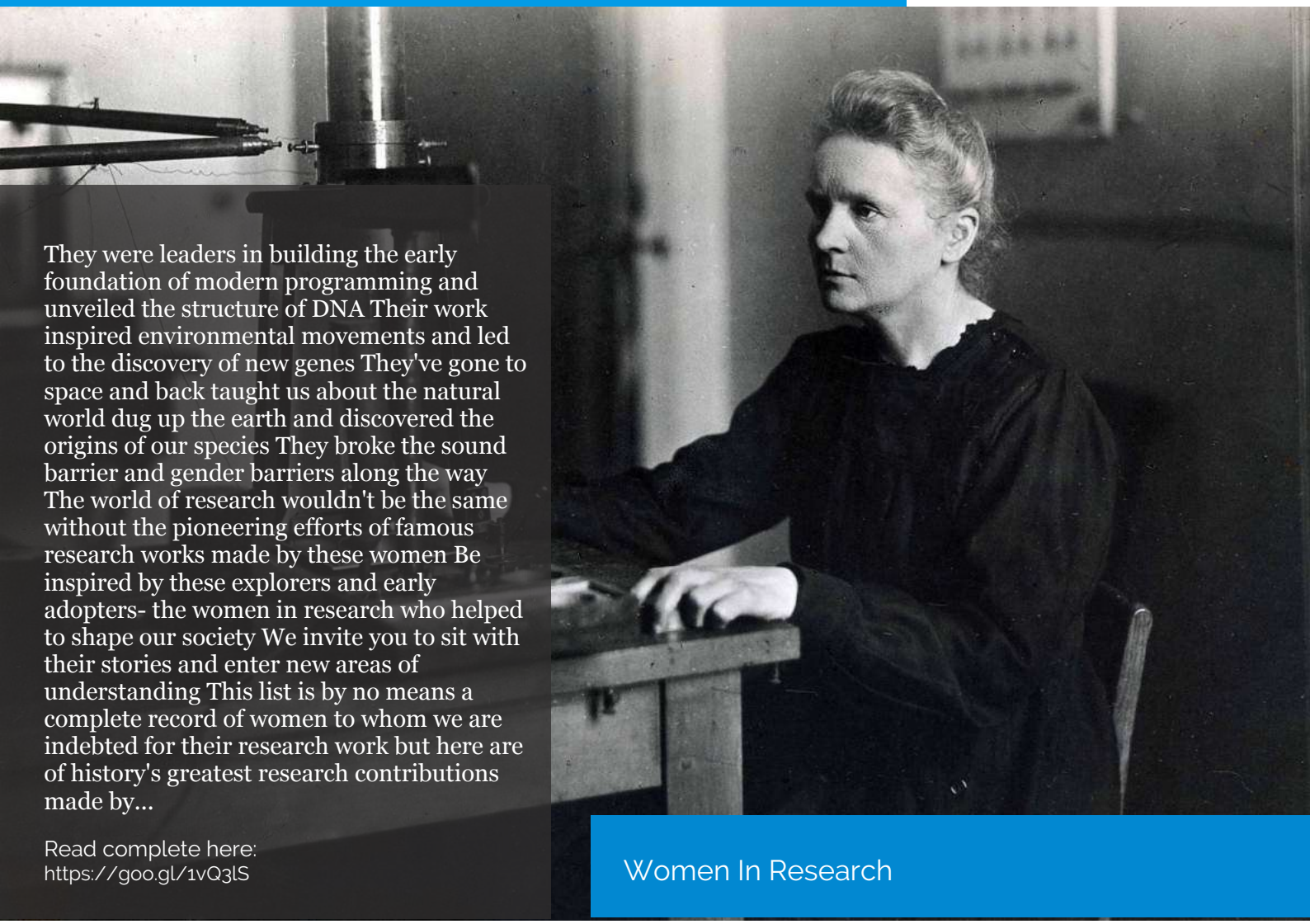

They were leaders in building the early foundation of modern programming and unveiled the structure of DNA Their work inspired environmental movements and led to the discovery of new genes They've gone to space and back taught us about the natural world dug up the earth and discovered the origins of our species They broke the sound barrier and gender barriers along the way The world of research wouldn't be the same without the pioneering efforts of famous research works made by these women Be inspired by these explorers and early adopters- the women in research who helped to shape our society We invite you to sit with their stories and enter new areas of understanding This list is by no means a complete record of women to whom we are indebted for their research work but here are of history's greatest research contributions made by...

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Preferential Induction of Canonical IMD and Toll Innate Immune Receptors by Bacterial Challenges in Triatoma Pallidipennis Primed with Gram-Negative and Gram-Positive Bacteria, Respectively

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ABSTRACT

Insects lack an adaptive immune defense against invading microorganisms, but they possess humoral and cellular response similar to that of vertebrates. The Immune Deficiency (IMD) and Toll are the major signaling pathways to produce humoral antimicrobial peptides AMPs. Pathogen molecular patterns (PAMs) of Gram-negative bacteria activate Pattern recognition receptors (PRR) of the IMD pathway, while PAMS of Gram-positive activate PRR of the Toll pathway.

Although the IMD pathways is incomplete in Hemipterans, in Triatoma pallidipennis, there is a preferential participation of the IMD pgrp-lc and toll receptors in the responses to Gram- negative and Gram-positive bacteria, respectively. Still, as in other insects, cross induction was observed.

Keywords: priming, *Triatoma pallidipennis*, tppgrp-lc, tptoll, IMD pathway, toll pathway.

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Although the IMD pathways is incomplete in Hemipterans, in Triatoma pallidipennis, there is a preferential participation of the IMD pgrp-lc and toll receptors in the responses to Gram-negative and Gram-positive bacteria, respectively. Still, as in other insects, cross induction was observed. An enhanced protection after a previous exposure to a pathogen, termed priming, functionally homologous to the adaptive immune memory of vertebrates, has been documented in several insect Orders but not in Hemiptera, and the participation of the components of the immune signaling cascades remains poorly explored. We present evidence for immune priming to Micrococcus luteus (Gram-positive) and Escherichia coli (Gram-negative) bacteria in T. pallidipennis. The preferential participation of receptors of the IMD and Toll pathways in the responses to each bacterial challenge was recorded.

Keywords: priming, *Triatoma pallidipennis*, tp*pgrp-lc*, tp*toll*, IMD pathway, toll pathway.

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I. INTRODUCTION

Insects lack an adaptive immune defense system against invading microorganisms but respond with humoral and cellular effector mechanisms whose components have counterparts in vertebrates' innate immune responses [1- 4]. These were mostly unraveled from studies in *Drosophila melanogaster* and *Aedes aegypti* [5 - 7]. The principal effectors comprise lectins, melanin, and antimicrobial peptides (AMPs) [8-13] produced mainly in the fat body, hemocytes and the digestive tract [14].

The Immune Deficiency (IMD) [15] and Toll [16] signaling pathways, whose counterparts in vertebrates are the tumor necrosis factor receptor (TNFR) and the interleukin-1 receptor (IL-1R), and the Toll-like receptors (TLRs) [17], respectively, are the major signaling pathways to produce AMPs [18]. Pathogen–associated molecular patterns (PAMPs) of invading microorganisms, that are recognized by pattern-recognition receptors (PRRs) [12, 13, 19, 20], activate these immune pathways. The canonical pathways were described mainly in the Dipterans *Drosophila* flies and *Aedes* mosquitoes.

Accordingly, Gram-negative bacteria are recognized by a transmembrane receptor that recruits and activates the IMD cascade leading to the expression of the AMP genes *cecropin, attacin, diptericin, drosomycin* [21, 22].

Gram-positive bacteria and fungi bound to proSpatzle induce its proteolytic cleavage, Spatzle

binds to the Toll receptor on the cell membrane [23], which in turn triggers the Toll cascade leading to the expression of the AMP genes *drosomycin, defensin B,* and *metchnikowin* [19, 24, 25). Although Gram-negative and Gram-positive bacteria preferentially activate IMD and Toll signaling pathways, respectively, there are cross interactions among these and other immune pathways such as JAK/STAT (26- 28], and they could be synergetic [29].

The Toll and IMD pathways are fully present in most insects [25], but this is not true for several Hemipterans, including reduvids. In this family, ortholog molecules, including the PRRs, *pgrp-lc* and *pgrp-la,* and AMPs have been identified [30-32]. In addition, there is evidence for the presence of key members of the IMD pathway (IMD and Relish) in *R. prolixus*, [34]. But genes coding this pathway appear incomplete or absent in *Triatoma dimidiata, Triatoma infestans and Triatoma pallidipennis* [31, 33]. Nevertheless, it was documented in *T. pallidipennis* the preferential participation of the IMD *pgrp-lc* and *toll* receptor genes and the *Relish* transcription factor in the regulation of responses to Gram-positive and Gram-negative infections, respectively, but as in other insects, cross induction was observed [35].

In contrast to vertebrates, the mechanisms for adaptation and selection of immune effector molecules are lacking in insects. However, enhanced protection after a previous exposure to a pathogen, termed immune priming [36, 37], has been documented in Diptera, Coleoptera, Lepidoptera, Homoptera, Hymenoptera, and Orthoptera (revised by Contreras *et al.* [38]). This immune priming is functionally homologous to the adaptive immune memory of vertebrates [36, 37, 39].

As no adaptive modifications of the protective molecules, such as AMPs occur, the molecular mechanisms participating in the induction of immune priming are mainly unknown. The increased response to a second encounter is associated with an increased production of immune effector molecules [40, 41]. Still, the participation of the components of the immune signaling cascades remains poorly explored [42].

We present herein evidence for immune priming responses to sublethal doses of *Micrococcus luteus* (Gram-positive) and *Escherichia coli* (Gram-negative) bacteria in *T. pallidipennis*, a primary vector of Chagas disease in Mexico [43].

In addition, we documented the preferential participation of the IMD and Toll immune pathways receptors in the responses to each bacterial challenge, respectively.

II. METHODS

2.1 Insects Rearing

Triatoma pallidipennis was obtained from a colony initiated with specimens collected in different locations in the Morelos State, Mexico. The colony was maintained in the insectary of the National Institute of Public Health of Mexico.

Fifth-stage nymphs were maintained at 28°C and 70–80% relative humidity under a photoperiod of 12 h light and 12 h dark. They were fed rabbit blood after molting, using artificial feeders. All experiments were conducted using 10-12 days-post-feeding-fifth-instar nymphs. The protocols were approved by the Biosafety, Ethics and Research Committees of the National Institute of Public Health (file number, CB17-229, CB:1491, CI:1500).

2.2 Cultures of Escherichia Coli and Micrococcus luteus

Gram-negative *Escherichia coli* (DH5α8739 strain atcc.org) was grown overnight at 37°C in Luria-Bertani Broth (Dibico, Mexico) with agitation. 100µL was added to 2mL of fresh medium in a test tube and incubated until reaching an optical density of 0.7 (O.D. 600 nm).

The culture was centrifuged at 8000 rpm at 4°C, and the pellet recovered in PBS-pH 7.4 (137mM NaCl, 2.7mM KCl, 10mM sodium phosphate). Bacteria concentrations were quantified by colony forming units (CFU) in Luria Bertani-Broth medium with 1.5% bacteriological agar (BD Bioxon, Becton Dickinson, Mexico). Lyophilized Gram-positive *Micrococcus luteus* (Sigma-Aldrich, M-0508) was diluted in 5mL of PBS-pH 7.4. The viability of the sample was determined in an aliquot of 10µL using Trypan Blue at 0.4% in

PBS. (Corning USA) and counted in a Neubauer chamber. Translucent white colonies were counted individually in an optical microscope (Olympus Optical Co, LTD, Japan) at 100X. Bacteria concentrations were determined by counting CFU of cultures in Luria Bertani-Broth medium with 1.5% bacteriological agar (BD Bioxon, Becton Dickinson, Mexico).

2.3 Infection of T. Pallidipennis with Gram-Negative and Gram-Positive Bacteria

Groups of ten fifth-stage nymphs, 10 to 12 days post-feeding, cold-anesthetized (4 °C), were injected through the cuticular inter-tegument between the head and the thorax with live 10^3 CFU of *M. luteus*, 10^6 CFU of *E. coli* in 20µL of PBS, control groups were inoculated with 20 µL of sterile PBS using a syringe (31GX8mm needle).

On 1-, 15-, and 21 days post-inoculation (priming), five specimens of each group were dissected, and their fat body tissues were recuperated. Tissue samples were stored in 200 µLTRIzol (Thermo Fisher Scientific, Waltham, MA, USA) at −70 °C until processing for quantitative real-time polymerase chain reaction (qPCR) of transcripts.

After 21 days post-inoculation, five to seven nymphs per group were challenged with the same quantities of *M. luteus*, *E. coli* bacteria or PBS as previously described. At one- and three- day post-challenge, the fat bodies of five cold-anesthetized specimens of each group were individually obtained in PBS. Tissue samples were stored in Eppendorf tubes in 200 µL TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) at −70 °C until processed for total RNA extraction. Each treatment had three replicates per group.

2.4 RNA Extraction and cDNA Synthesis.

Total RNA from fat body tissues was extracted using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's recommendations. Briefly, about 50 mg of each fat body sample in 200 µL TRIzol in Eppendorf tubes (Thermo Fisher Scientific) were macerated using a biovortex. After adding 40 µL of chloroform (Sigma- Aldrich, St. Louis, MO, USA), the preparations were mixed and centrifuged for 15 min at 10,000g at 4 °C. The aqueous phase was recovered, and 200 µL of cold isopropanol (Sigma-Aldrich) was added, mixed, and incubated at −20 °C for one h. The samples were centrifuged at 10,000g for 10 min, and the pellets were washed with 500 µL 75% ethanol and centrifuged at 10,000g for 15 min (Scilogex, D3024R centrifuge). The supernatants were removed, and the pellets were suspended in 40 µL diethyl pyrocarbonate (DEPC, Sigma-Aldrich)-treated water. RNA was quantified with a NanoDrop 1000 spectrophotometer v. 3.7 (Thermo Fisher Scientific) and visualized using electrophoresis in agarose gels stained with EpiQuik DNA stain (EpiGentek, Farmingdale, NY, USA). Ten micrograms of total RNA were treated with 1 unit of DNAse I (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at 37 °C, and subsequently inactivated with 1uL 25mM EDTA (ethylenediaminetetraacetic acid) at 75°C for 15 min.

First-strand cDNA synthesis was performed in 25 µL reactions containing 10 µg total RNA using an oligo dT 18 mero primer (Thermo Fisher Scientific) with 200 units of Reverse Transcriptase enzyme IV (SuperScript ®, Thermo Fisher Scientific). The synthesis reactions were incubated for one h at 42 °C, and then inactivated at 75°C for 15 min. The synthesized cDNA was diluted 1:10 with DEPC water and stored at −70 °C until use.

2.5 PCR of Tppgrp-lc, Tptoll, and AMP Transcripts

The transcription of Tp*pgrp-lc*, Tp*toll*, Tp*defensin B,* and Tp*prolixicin* was confirmed in cDNA templates by RT-PCR, using the *T. pallidipennis* *β*-actin gene as control. Oligonucleotides sequences and PCR reaction conditions are reported in [35].

2.6 Quantitative PCR

We used qPCR to analyze the expression of Tp*pgrp-lc* and Tp*toll* receptors and *prolixicin* and *defensin B* transcripts in individual cDNA samples of fat body tissue after the priming and challenge with *M. luteus* and *E. coli* as previously reported [35]. Briefly, each reaction was performed in a final volume of 10 µL, containing 2 µL of cDNA (1:10), 1.5 pmol of each oligonucleotide, and 5 µL of SYBR Green 2X Mix (NZY qPCR Green Master

Mix, nzytech, Lisbon, Portugal). qPCR was performed in a Rotor-Gene Q 5plex (Qiagen, Hilden, Germany). The qPCR conditions used were as follows:50°C for 2min, 95°C for 5 min, 35 cycles of 95 °C for 20s and 65 °C for 60s. A melt curve analysis was conducted to confirm the specificity of the reaction. Controls without templates were included with each primer set to verify the absence of exogenous DNA and oligonucleotide dimers.

2.7 Statistical Analysis

The relative differences in the expression of transcripts were calculated using the 2−ΔΔCt method [44]. As endogenous control, we used the *β-actin* gene. The values obtained from the ΔCt analysis (Ctvalue of problem transcript – Ctvalue of *β-actin*) were used to compare each transcript between groups (ΔΔCt) in all experiments (e.g., ΔCprolixicin priming group – Δcprolixicin PBS group). ANOVA not RM tests were performed to determine differences in gene expression between each treatment and their controls adjusted by Tukeys analysis. Graphs were made using GraphPad Prism 6. *P*-values of $P < 0.05$ were considered significant.

III. RESULTS

The expression of Tp*pgrp-lc*, Tp*toll*, Tp*defensin B*, and Tp*prolixicin* was estimated by PCR in the fat body of specimens of all experimental groups. Inoculation with *E. coli* and *M. luteus* increased the transcription of immune response genes, and significant differences were observed in some transcripts after the first bacterial infections and after challenges.

Tprolixicin increased significantly in insects infected (priming) with *E. coli* (4.08-fold, SE 2.00–6.15) on day one after priming, and its expression was reduced on days 15 to 21 after priming, but it increased to levels higher than those of the priming response (13.68-fold, SE 7.67-20.82– $P = 0.0146$ and 51.19-fold, SE 19.18-149.08 $P = 0.0064$) on days 1 and 3 respectively after challenge with the same bacteria. (Fig. 1A, Table1).

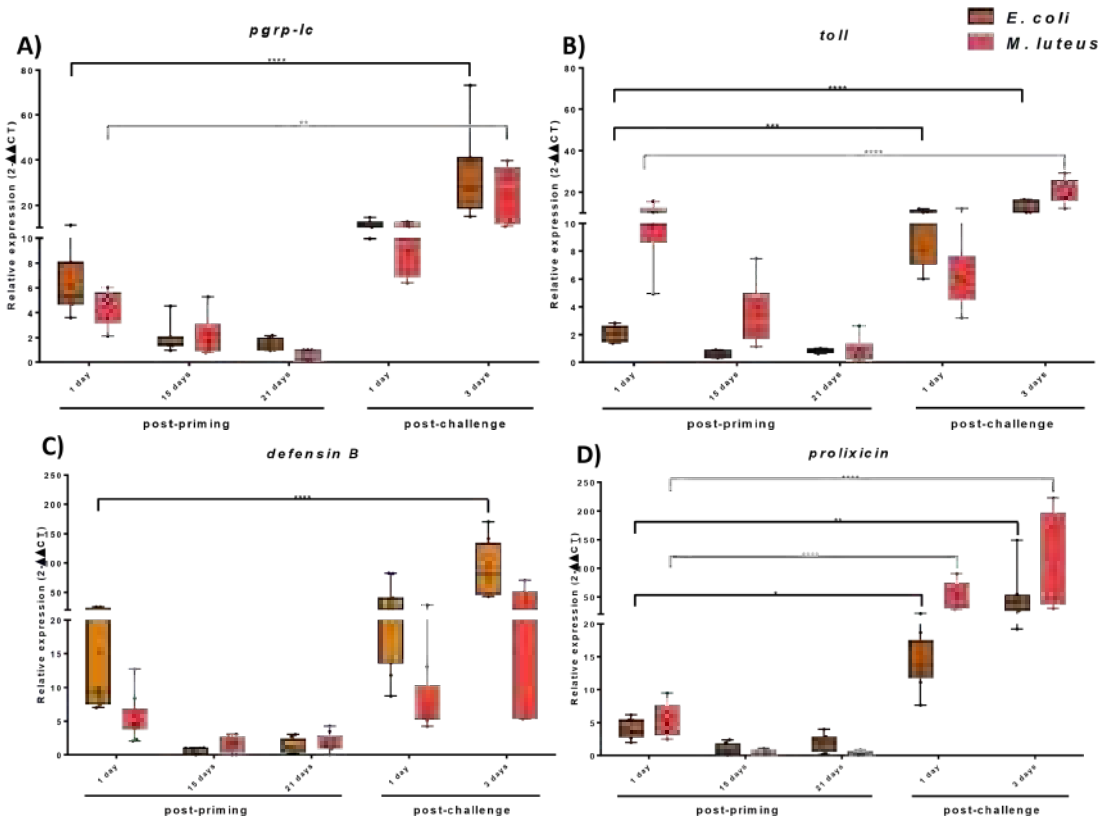

Figure 1: Relative expression of Tp*prolixicin (A),* Tp*defensin B* (B), Tp*pgrp-lc* (C), *and* Tp*toll* (D) transcripts in fat body of priming and challenge *T. pallidipennis* with *E. coli* or *M. luteus*. In priming bugs, Tp*prolixicin* and Tp*toll* transcripts increased against both bacteria after a day post-priming and these increase was greater against *M.*

luteus after one and three-days post-challenge (A and D). Similar results were observed in Tp*defensin B* and *Tppgrp-lc*, but the transcripts expression was greater against *E. coli* (B and C). Relative expression (2−ΔΔCT) is the quantified change of expression between transcripts, asterisks indicate significative *p value* (*$p = 0.0146$, ** $p = 0.0064$, *** $p = 0.0030$ and **** $p < 0.0001$), bars represent the mean transcript levels ± 95% CI. Groups were normalized with unchallenged and pbs group adjusted for *β-actin*.

Table 1: Comparasion of the relative expression of Tp*prolixicin*, Tp*defensin B*, Tp*prgp-lc* and Tp*toll* transcripts during the first bacterial infection (1-day post priming) and the second bacterial infection (1 and 3 days post challenge). Priming values are shown in gray, challenge values with increased expression are shown in green and those without significant increase are shown in red. All values are shown in fold change. Standard errors and stadistical significance are shown in parentheses.

	1 day post-priming	15 days post-priming	21 days post-priming	1 day post-challenge	3 days post-challenge
defensin B vs *E. coli*	13.36 (7.03-25)	0.69 (0.010-1.020)	1.27 (0.15-3.0)	34.0 (8.69-82.70, ns $p > 0.05$)	90.29 (43.10-170, $p < 0.0001$)
defensin B vs *M. luteus*	5.48 (2.07-12.70)	1.49 (0.01-3.02)	1.80 (0.04-4.28)	9.21 (4.25-28.24, ns $p > 0.05$)	26.73 (5.33-70.52, ns $p > 0.05$)
prolixicin vs *E. coli*	4.08 (2.0-6.15)	0.98 (0.040-2.39)	1.89 (0.13-4.0)	13.68 (7.67-20.82, $p = 0.0146$)	51. 19 (19.18-149.08 $p = 0.0064$)
prolixicin vs *M. luteus*	5.27 (2.46-9.45)	0.51 (0.11-1.08)	0.30 (0.06-0.86)	51.40 (27.85-90.50, $p < 0.0001$)	112.81 (29.44-222.86, $p < 0.0001$)
pgrp-lc-E. coli	6.31 (3.60-11.10)	1.87 (0.95-2.06)	1.49 (0.95-2.15)	11.51 (9.95-14.52, ns $p > 0.05$)	33.78 (14.92-73.00, $p < 0.0001$)
pgrp-lc vs *M. luteus*	4.47 (2.14-6.03)	2.07 (0.80-5.27)	0.60 (0.15-1.00)	9.61 (6.40-12.50, ns $p > 0.05$)	25.97 (12.29-39.65, $p = 0.0064$)
toll vs *E. coli*	2. 07 (1.40-2.84)	0.61 (0.35-0.90)	0.85 (0.65-1.00)	9.42 (6.02-12.01, $p = 0.0003$)	12.98 (10.28-16.28, $p < 0.0001$)
toll vs *M. luteus*	12.52 (4.92-15.60)	4.12 (1.14-7.46)	1.04 (0.11-2.63)	7.63 (3.22-12.14, ns $p > 0.05$)	24.02 (17.80-29.12, $p < 0.0001$)

Like the infection with *E. coli*, the infection (priming) with *M. luteus* resulted in significant increases in the expression of *Tprolixin* (5.27-fold, 2.46-9.45), but its expression was more significant after 1 and 3 days of the challenge (51.40-fold, SE 27.85-90.50 $P < 0.0001$ and 112.81-fold, SE 29.44–222.86 $P < 0.0001$), respectively (Fig.1A, Table1). The increase in the levels of Tp*prolixin* transcripts induced by the challenge with *M. luteus* was higher than those by the challenge with *E. coli* (Fig. 2, Table 2).

Priming with *E. coli* produced an increase of Tp*defensin B* transcripts on day one post-priming (13.36-fold, SE 7.03-25), and these decreased at days 15 to 21 post-priming, but the challenge with the same bacterium produced increased levels of the transcript (34-fold, SE 8.69–82.70 $P > 0.05$ and 90.29-fold, SE 43.10–170 $P < 0.0001$) on days 1 and 3 post-challenge with the same bacterium, respectively (Fig. 1B, Table 1).

Priming with *M. luteus* resulted in a moderate increase of Tp*defensin* (5.48-fold, SE 2.07–12.70) on day one post-priming, and its level decreased on day 15 and remained low until day 21 post. priming. Challenge with the same bacterium did not induce statistically significant increases on days 1 and 3 post-challenge (9.21-fold, SE 4.25–28.24 $P > 0.05$ and 26.73-fold, SE 5.33-70.52 $P > 0.05$). However, the increases in Tp*defensin B* transcripts induced by the challenge with *E. coli* were higher than those by the challenge with *M. luteus* (Fig. 2, table 2).

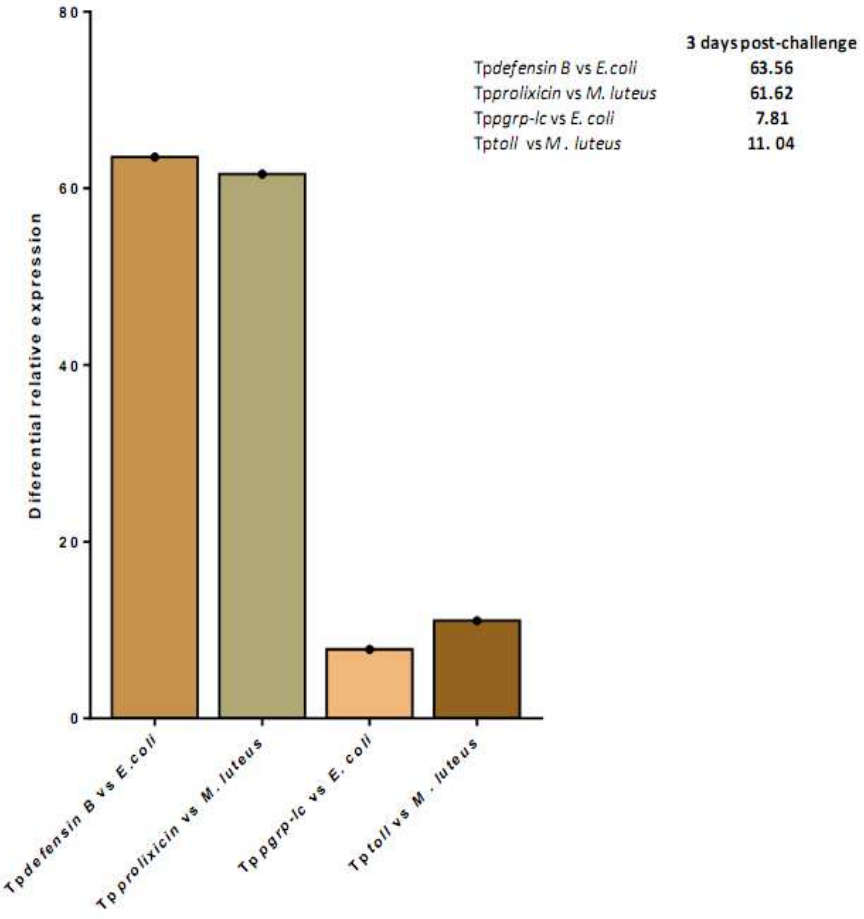

Figure 2: Differential relative expression of AMPs and receptors of *T. pallidipennis* after three days post-challenge with *E. coli* or *M. luteus*. Tp*defensin B* and Tp*pgrp-lc* transcripts were more intensely expressed when inoculated with *E. coli*, while Tp*prolixicin* Tp*toll* were more expressed in response to *M. luteus*.

Table 2: Differential expression of AMPs, and Tp*pgrp-lc* and Tp*toll* receptors during the 1-and 3-days post challenge with *E. coli* or *M. luteus*. *Tpdefensin B* and *Tppgrp-lc* were more expressed against *E. coli*, while Tp*prolixicin* and Tp*toll* against *M. luteus***.** The differential values with increased expression are shown with an asterisk. Differential expression was obtained by comparing the values of a specific transcript in response to infection of each bacterial species. The values with increased expression are shown in green and those with no significant increase are shown in red. All values are shown in fold change.

	1 day post-challenge	3 days post-challenge		1 day post-challenge	3 days post-challenge
Tp*defensin B* vs *E. coli*	34	90.29	Tp*prolixicin* vs *E. coli*	13.68	51. 19
Tp*defensin B* vs *M. luteus*	9.21	26.73	Tp*prolixicin* vs *M. luteus*	51.4	112.81
	24.79	63.56*		37.72*	61.62*
Tp*pgrp-lc* vs *E. coli*	11.51	33.78	Tp*toll* vs *E. coli*	9.42	12.98
Tp*pgrp-lc* vs *M. luteus*	9.61	25.97	Tp*toll* vs *M. luteus*	7.63	24.02
	1.9	7.81*		1.79	11.04*

	3 days post-challenge	1 day post-challenge
Tp*defensin B* vs *E. coli*	63.56*	24.79
Tp*prolixicin* vs *M. luteus*	61.62*	37.72*
Tp*pgrp-lc* vs *E. coli*	7.81*	1.9
Tp*toll* vs *M . luteus*	11. 04*	1.79

Tp*pgrp-lc* increased after infection with *E. coli* (6.31-fold, SE 3.60–11.10) but transcript levels decreased on days 15 and 21. After challenge with the same bacterium, Tp*pgrp-lc* increased significantly on three days post inoculation, (33.78-fold, SE 14.92-73 $P < 0.0001$) (Fig. 1C, Table1).

After priming with *M. luteus,* increases of Tp*pgrp-lc* levels were observed on day one post-inoculation (4.47-fold, SE 2.14-6.03), and the transcript levels remained low up to 21 days. After a challenge with the same bacterium, transcription increased at levels higher than those observed during the first infection. Increased levels were higher after three days post-challenge (25.97-fold, SE 12.29–39.65 $P < 0.0064$). On day one, no statistically significant increases were observed (9.61-fold, SE 6.40–12.50 $P > 0.05$). The increase in Tp*pgrp-lc* transcripts induced by the challenge with *E. coli* was higher than those by the challenge with *M. luteus*.

Tp*toll* transcripts increased after priming with *E. coli* (2.07-fold, SE 1.40-2.84) levels below those of pre-priming were observed on days 15 and 21. After challenge, Tp*toll* transcripts increased (9.42-fold, SE 6.02–12.01 $P = 0.0003$ and 12.98-fold, SE 10.28-16.28 $P < 0.0001$) on the first- and third- day post-injection, respectively. Using *M. luteus,* Tp*toll* transcripts increased during the priming (12.52-fold, SE 4.92-15.60), and the challenge with the same bacterium resulted in a new rise in *TpToll*, reaching higher levels that those observed after the first infection (24.02-fold, SE 17.80–29.12 $P < 0.0001$) on day three post-challenge. The increase in the levels of Tp*toll* transcripts induced by the challenge with *M. luteus* was higher than those by the challenge with *E. coli* (Fig. 2).

IV. DISCUSSION

We documented in *T. pallidipennis* the participation of *Tppgrp-lc* and Tp*toll* receptors in the induction of immune priming, and in the enhanced immune response after a second challenge with the same bacteria. In addition, we confirmed our previous observations on the preferential participation of these receptors in the recognition and induction of the primary immune innate response to bacterial infections.

As we employed sub-lethal doses of *E. coli* and *M. luteus* for the challenge, we could not assess any possible protection effect of the priming induced by first bacterial infections. Nevertheless, the *defensin B* and *prolixin* transcription responses after a second bacterial challenge fulfilled the essential parameters of priming: specificity [36] and long-lasting biphasic response [37, 39]. However, in analyzing the molecular specificity of the reactions, some precisions are required. Although the infection with Gram-positive and Gram-negative bacteria induced the transcription of both AMPs, and transcription levels decreased with time in both infections. The Tp*defensin B* initial response was higher after infection with *E. coli* than with *M. luteus*, confirming the preferential induction of the IMD pathway by the Gram-negative bacterium. The response to *M. luteus* could indicate possible Tp*pgrp-lc* isoforms responding to Gram-positive bacteria, or the direct induction of components of the IMD pathway. Similar primary responses were observed in *D. melanogaster* [21, 26]

A second challenge with *E. coli* resulted in higher levels of Tp*defensin B* transcript, while a second challenge with *M. luteus* did not induce significant changes in its transcription. After priming, the differences between the responses support a preferential response to the Gram-negative bacterium, which is in accord with a preferential IMD pathway induction. Although we did not explore possible changes in the transcription of other components of the IMD pathway, changes in *Tppgrp-lc* transcription after challenge with *E. coli* followed a similar pattern to that of Tp*defensin B*. In contrast, after challenge with *M. luteus,* this was not significant. We speculate that the increased Tp*pgrp-lc* receptor, augmented the recognition of the *E. coli* used for challenge, thus the enhanced Tp*defensin B* response. This confirms a relative degree of the specificity of the priming response within the cross interacting IMD and Toll system, a condition favouring a broad-spectrum innate Immune response [21].

On the other hand, *Tprolixin* and Tp*toll* transcription levels increased after the first

infection with both bacteria, but they were higher after infection with *M. luteus*. The participation of Tp*toll* and other Toll receptors in responses to Gram-positive, indicates higher receptor affinity to the Gram-positive bacterium. Toll pathway induction by Gram-positive and Gram-negative bacteria was observed in other insects [45, 46] and could explain our observations. In the same way, the transcription of *Tprolixin* and Tp*toll* was higher after a second challenge with both bacteria, but the challenge with *M. luteus* resulted in higher transcription levels than with the challenge with *E. coli*. The increase of the Toll receptor after priming could explain the enhanced response to *M. luteus* during the challenge.

Our observations reflect the diversity in the induction of the innate immune pathways among insects. It could be explained by cross-interactions between the members of two central innate immune cascades. The capacity of the receptors to interact with diverse molecules in Gram-positive and Gram-negative bacteria is shown by diverse toll receptors that could mediate the activation of immune responses against Gram-negative bacteria [4]. On the other hand, as bacteria contain a mixture of antigens, it is possible that although Gram-negative bacteria preferentially induce the IMD immune system and Gram-positive the Toll system, some bacterial antigens may induce the other immune cascade than the canonically, up to now accepted. Nevertheless, the higher responses after the challenge are in order with the specificity of the priming inductor.

The mechanisms underlying immune priming remain poorly understood; however, specific receptor induction is expected for specific responses. Specific, or in the case of *T. pallidipennis* preferential, induction began with activating a particular receptor. Our results indicate that transcription of *Tppgrp-lc* and Tp*toll* receptors increased after the first exposition to their respective bacterium, indicating that a mechanism activates the production of more receptors than those already present before exposition. Similar results were observed in *Anopheles gambiae* exposed to bacteria [47].

Increased numbers of receptors could be responsible of the primary response,and having already more receptors could explain an increased response to the second exposure. As stablished in canonical priming (biphasic response), the transcription effectors Tp*prolixin* and Tp*defesin B* subdued after three weeks, so did their receptors, probably because exhaustion of the respective antigens. Interestingly, an even higher receptors' transcription occurred after the challenge. This indicates possible not yet defined mechanisms responsible for induction of the transcription during the first pathogen encounter, specifically (or preferentially) triggered again with the challenge.

We did not explore the participation of other members of the IMD and Toll immune cascades that could play a role in directly modulating the immune response at the transcription level. There is evidence in *Drosophila* that Toll, but not IMD, is necessary, although insufficient for priming induction (48), but its participation still requires revealing. Epigenetic DNA/RNA dynamic modifications by methylation could regulate gene expression [49], possibly explaining "memory", but the underlaying mechanism for enhancement is not responded. Endoreplication (DNA synthesis without cell division) has been documented in *An. Albimanus* infected with *Sacharomyces cerevisiae* [50] and *Plasmodium berghei,* probably increasing the number of immune genes copies during the first encounter with the pathogens. This, along with the transcription of regulatory elements of the cell cycle [51], could indicate the involvement of enhanced transcription in the immune response but does explain further enhancement after challenge.

In conclusion, we have documented in the Hemipteran T. pallidipennis the response to Gram-negative and Gram-positive bacteria with the main features of priming: biphasic and enhanced AMPs transcription response after a second challenge. Both phases were associated to the preferential induction of IMD and Toll receptors, respectively. The mechanisms underlying the induction of their transcription warrant further research.

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The authors declare that they have no conflict of interest related to the findings or techniques in the present study.

List of abbreviations:

qPCR (quantitative polymerase chain reaction)
pgrp-lc (Peptidoglycan recognition protein-long chain)
AMPs (Antimicrobial peptides)
IMD (Immune deficiency signalling pathway)

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ABSTRACT

Carbapenem-resistant Escherichia coli and Klebsiella pneumoniae are increasingly important pathogens with limited treatment options, and there is limited knowledge on the environmental factors contributing to their spread. We determined the occurrence of carbapenem- resistant E. coli and K. pneumoniae in hospital and slaughterhouse wastewater in Owerri, a city in southeast Nigeria. Samples of untreated hospital and slaughterhouse waste- water were collected monthly at the major tertiary hospital and slaughterhouse in Owerri between April and September 2023. E coli and K. pneumoniae strains were selectively isolated and identified using conventional microbiological technique, and antibiotic susceptibility testing performed using the Kirby Bauer disk diffusion assay. A total of 193 E. coli and K. pneumoniae were isolated from the 269 wastewater samples analyzed. Among the 193 isolates, 101 (52.3%) were identified as K. pneumoniae while 92 (45.7%) were identified as E. coli respectively.

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ABSTRACT

Carbapenem-resistant Escherichia coli and Klebsiella pneumoniae are increasingly important pathogens with limited treatment options, and there is limited knowledge on the environmental factors contributing to their spread. We determined the occurrence of carbapenem- resistant E. coli and K. pneumoniae in hospital and slaughterhouse wastewater in Owerri, a city in southeast Nigeria. Samples of untreated hospital and slaughterhouse waste-water were collected monthly at the major tertiary hospital and slaughterhouse in Owerri between April and September 2023. E coli and K. pneumoniae strains were selectively isolated and identified using conventional microbiological technique, and antibiotic susceptibility testing performed using the Kirby Bauer disk diffusion assay. A total of 193 E. coli and K. pneumoniae were isolated from the 269 wastewater samples analyzed. Among the 193 isolates, 101 (52.3%) were identified as K. pneumoniae while 92 (45.7%) were identified as E. coli respectively.

The isolates had high resistance rates (≥48.1%) to 6 antibiotics tested, and resistance to carbapenems ranged from 22.8% to 30.6% with resistance to ertapenem, 30.6 %(59/193) being the highest carbapenem resistance observed. There was no statistical significant difference in carbapenem resistance rates between the hospital and slaughterhouse wastewater isolates (P > 0.05). This study has shown that the release of untreated wastewater into the environment may contribute to the increased spread of carbapenem-resistant E. coli and K. pneumoniae in Owerri, Nigeria. Therefore, there is pressing need to address wastewater as a crucial factor in curtailing the spread of carbapenem-resistant bacteria.

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I. INTRODUCTION

Untreated wastewaters from hospitals, agriculture, slaughterhouses, industry and, together with domestic wastewaters contribute to the contamination and degradation of aquatic environments (Oliveira et al., 2021). The release of untreated or poorly treated wastewater into the environment is being increasingly recognized as the major factor in spreading clinically relevant antimicrobial resistant bacteria and genes (Obayiuwana and Ibekwe, 2020). Antimicrobials in wastewater promote the emergence of antibiotic resistance, facilitated by selective pressure and transfer of resistant genes. The widespread use of antimicrobials in clinical practice to control infectious diseases, their application in veterinary medicine coupled with the discharge of non-treated pharmaceutical effluent into the environment, results in the selective pressure which is associated with the emergence and subsequent evolution of bacterial resistant to antibiotics (Tiwari et al ., 2022).

Contamination of water bodies is dependent on the amount, type and degradation potential of contaminants, and the self-purification ability of the recipient water body (Obasi et al 2017). Human and animal pathogens are considered to be important water contaminants; this is possible because, together with human and animal pathogens from all infected individuals and animals (symptomatic, asymptomatic, pre-symptomatic, and post-symptomatic), is excreted through feces, urine, nasal mucus, and sputum

from veterinary clinics, slaughterhouses, households, hospitals, and nursing homes, end up in the municipal sewage system. Within the concept of one health perspective, such discharges of clinically relevant antibiotic-resistant bacteria pose a significant health risk to both humans and animals. Therefore, determining the prevalence of clinically important antibiotic resistant bacteria in wastewater may help in controlling their circulation in the environment and saving human and environmental health.

Carbapenems are broad range β-lactam antibiotics used as a last resort in treating infections with multi-drug resistant *Enterobacteriaceae* (MDR-E). In the past few decades, the emergence of strains resistant to carbapenems has been a growing concern (Meletis, 2016). In February 2017, the World Health organization (WHO) published a list of the most dangerous pathogens for human health. At the top of that list are carbapenem-resistant *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacteriaceae* (WHO, 2017). There have been several reports of bacteria with acquired carbapenem resistance isolated from hospital wastewaters (Zhang et al., 2013; Chandran et al., 2014; Seruga-Music et al., 2017), as well as raw and secondary treated municipal wastewaters (Hrenovic et al., 2017). However, data on the prevalence of bacteria of clinical importance from wastewater sources in southeast Nigeria is scarce; most studies are centered on the clinical environment and there is no information about prevalence of carbapenem-resistant *E. coli* and *K. pneumoniae* in environmental sources in the region. This study was designed to bridge this knowledge gap and was therefore, aimed to investigate the prevalence of *E. coli* and *K. pneumoniae* isolated from hospital and slaughterhouse wastewater sources in Owerri, a city located in southeast Nigeria, during a regional surveillance program conducted in March to September 2023.

II. METHODOLOGY

2.1 Sample Collection and Analysis

Untreated wastewater samples were drawn from the Federal University Teaching Hospital Owerri wastewater discharge points and a discharge point of Owerri slaughterhouse. Samples were collected from the upper 5 cm portion of each of the two wastewater sources in triplicate and pooled to form a composite sample. The samples were taken aseptically in sterile plastic bottles under refrigerated conditions and processed in the laboratory within 4 h after collection. Wastewater samples were serially diluted with peptone water and a 100 µl volume of each dilution concentration (original, 1/10, 1/100, and 1/1000) was inoculated on MacConkey agar with a spread plate technique, following the manufacturer's instructions. The plates were incubated at 37°C for 18–24 h, after which colonies were counted and expressed as colony forming units (CFU) per milliliter (ml). Colonies resembling *E. coli* and *K. pneumoniae* were sub-cultured in nutrient agar and the isolates were identified using conventional microbiological techniques.

2.2 Antibiotics Susceptibility Testing

The antibiotic susceptibility pattern of the isolates was determined using the Kirby-Bauer Disk diffusion method on Mueller Hinton agar (Oxoid, England). Inhibition zone diameter values were interpreted using standard recommendations of the Clinical Laboratory Standard Institute (CLSI, 2012). Susceptibility was tested against ertapenem, imipenem, meropenem, amoxicillin/clavulanic acid, ceftazidime, and cefuroxime (Oxoid, England). *E. coli* ATCC 25922 was included as a reference strain. Isolates found to have an ertapenem, meropenem or imipenem ≤22mm inhibition zone diameter were classified as carbapenem-resistant isolates.

III. RESULTS

3.1 Prevalence of Bacterial Isolates

In this study, 269 wastewater samples from Owerri Nigeria were processed for carbapenem-resistant *E. coli* and *K. pneumoniae* isolation. The samples included 135 hospital wastewater and 134 slaughterhouse wastewater respectively. In all, a total of 193 bacteria comprising of consecutive 92 (46.7%) *E. coli* and 101 (52.3%) *K. pneumoniae* was isolated. One bacteria isolate was selected from one wastewater sample and one sample;

either hospital or slaughterhouse wastewater was collected and not both. Sample collection was based on availability and we could not compare isolation rate from hospital or slaughterhouse wastewater The distribution of the 92 *E. coli* isolates according to sample sources showed that 59 (64.1%) came from hospital wastewater while 33 (35.9%) came from slaughterhouse wastewater Similarly, 61.4% (62/101) of the 101 *K. pneumoniae* isolates were from hospital wastewater while 38.6% (39/101) of the isolates came from slaughterhouse wastewater respectively Table 1.

Table 1: The Overall Growth and Distribution of *Escherichia Coli* and *Klebsiella Pneumoniae* Within the Different Wastewater Sampled

Sample Source	Total number of Samples	Escherichia Coli	Klebsiella Pneumoniae	Total
Hospital Wastewater	135	59 (64.1%)	33(35.9%)	92(46.7%)
Slaughterhouse Wastewater	134	62 (61.4%)	39 (38.6%)	101(52.3%)
Total	269	121(62.7%)	72(37.3%)	193(71.7%)

3.2 Antimicrobial Susceptibility Testing

As shown in Table 2, high antibiotic resistance rates were observed in the isolates, no isolate was susceptible to all the antibiotics tested. Among the carbapenems, moderate to high resistance rate was observed; ranging from 22.8% in imipenem to 30.6% in ertapenem. As expected, very high resistance rate was also observed in cephalosporins including 55.9% in cefuroxime and 58.0% in ceftazidime respectively. Amoxicillin clavulanic acid, an antibiotic used for phenotypic detection ESBL production was considerably inactive against the isolates with a 41.9% resistance rate.

There was no statistically significant difference in carbapenem resistance rates between the hospital and slaughterhouse wastewater isolates ($P > 0.05$).

Table 2: Antibiotics Susceptibility Patterns of *Escherichia Coli* and *Klebsiella Pneumoniae* Isolates against the 6 Antibiotics Tested

Antibiotics	Number of Resistance	Number of E-Coli	Number of K. Pneumoniae,
Imipenem	44/193(22.8%)	32/44 (72.7%)	12/44(27.3%)
Meropenem	56/193(29.1%)	27/44(48.2%)	29/44(51.8%)
Ertapenem	59/193(30.6%)	39/44(66.1%)	20/44(33.9%)
Amoxicillin clavulanic acid	81/193(41.9%)	37/44(45.7%)	44/44(54.3%)
Cefuroxime	108/193(55.9%)	51/44(47.2%)	57/44(52.8%)
Ceftazidime	112/193(58.0%)	58/44(51.8%)	54/44(48.2%)

IV. DISCUSSION

Antibiotics resistance profiling of pathogens helps to identify the emergence of rare or new resistance threats and prioritize possible actions to be taken against them. The analysis of wastewater can reveal the circulation of antibiotic resistant bacteria and antibiotics resistance genes among the catchment communities. In this study, we carried out untreated wastewater surveillance for carbapenem resistance traits among *E. coli* and *K. pneumoniae*. Our results indicate that carbapenem-resistant *E. coli* and *K. pneumoniae* are widely distributed among the wastewater sources. The isolates expressed highest resistance to ertapenem (30.6%) while the least resistance was observed in imipenem with 22.8% resistance rate. To our knowledge, these are the earliest carbapenem-resistant *E. coli* and *K. pneumoniae* detected in non-clinical samples in Owerri southeast Nigeria. In agreement with our result, a study conducted in 2020 also reported carbapenem-resistant traits as the most abundant resistant traits in hospital wastewater in Helsinki (Majlander *et al.*, 2021). However, their study included genotypic characterization while ours was based on only phenotypic characterization due to limited resources. Another study from the Netherlands, based on molecular characterization

reported carbapenem-resistant genes from both hospital and municipal wastewater, but the abundance in hospital wastewater was higher than in the municipal wastewater (Buelow et al., 2018). As untreated wastewater is considered to provide a glimpse of the antibiotic resistant bacteria and antibiotic resistance genes circulating in a community, our findings indicate that ertapenem resistant *E. coli* and *K. pneumoniae* was the most prevalent carbapenem-resistant trait in Owerri during the study period.

The World Health Organization (WHO) has classified carbapenem-resistant *Enterobacterales* as a critical priority, driving the need to develop new antibiotics (Davies and Simeon, 2017). *E. coli* and *K. pneumoniae* isolates identified as carbapenem-resistant in this study have been described in the environment and reported to be potential clinical pathogens (Li et al., 2019).

Perhaps, some carbapenemase genes are related to aquatic environments and only occasionally cause infection. Many of these bacterial species could be asymptomatically carried by humans in their intestines, and thus be found in wastewater.

Clinical isolates can differ from wastewater-based isolates. The clinical isolates are pathogenic, but wastewater-based isolates better represent symbiotic and normal human bacteria. The presence of high concentrations of carbapenem resistant *E. coli* and *K. pneumoniae* isolates in both the hospital and slaughterhouse wastewater samples is not only a reflection of the increasing use of carbapenems in Nigeria, but also an additional evidence regarding the inefficiency of the conventionally applied wastewater treatments in the elimination of these microorganisms from the treated effluents, subsequently leading to their release into the environment (Oliveira et al., 2020).

V. CONCLUSION

The analysis of the antibiotic resistance phenotypes of the potentially pathogenic carbapenem resistant *E. coli* and *K. pneumoniae* isolated from hospital and slaughterhouse wastewater samples revealed that all were multidrug resistance, showing resistance phenotypes to more than three antibiotic classes.

The findings of this study, together with the clinical reports from the previous studies indicate a steady increase in carbapenem resistant bacteria in Nigeria and support wastewater surveillance as a potential preparedness tool for antimicrobial resistance surveillance. Wastewater surveillance could supplement the clinical surveillance approach in monitoring the possible circulation of antimicrobial resistance in communities and shows potential as a public health tool. Future studies could cover larger geographical areas of Nigeria and include molecular tools to obtain a more representative national picture. The detection of carbapenem resistant *E. coli* and *K. pneumoniae* in the two wastewater samples underlines the importance of proper wastewater treatment to avoid the dissemination of antimicrobial resistant bacteria in the environment and reduce public health risks.

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Why Citicoline (A Medical Food) Should Not be Prescribed to Treat People with Acute Ischemic Stroke: The Certainty of the Evidence

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ABSTRACT

Background: Citicoline, a medical food prescribed for ischemic stroke, faces scrutiny due to its unproven efficacy and potential harms. This essay, drawing on a recent Cochrane review and focusing solely on all-cause mortality, advocates for a critical reevaluation of its use. Rather than offering an updated Cochrane review, this analysis provides a reflective perspective through the lens of Evidence-based Medicine and Philosophy of Science.

Question Research: Why citicoline (a medical food) should not be prescribed to treat people with acute ischemic stroke: The certainty of the Evidence.?

Keywords: citicoline; medical food; acute ischemic stroke; evidence-based medicine; philosophy of science.

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ABSTRACT

Background: Citicoline, a medical food prescribed for ischemic stroke, faces scrutiny due to its unproven efficacy and potential harms. This essay, drawing on a recent Cochrane review and focusing solely on all-cause mortality, advocates for a critical reevaluation of its use. Rather than offering an updated Cochrane review, this analysis provides a reflective perspective through the lens of Evidence-based Medicine and Philosophy of Science.

Question Research: Why citicoline (a medical food) should not be prescribed to treat people with acute ischemic stroke: The certainty of the Evidence.?

Objective: Demonstrate from evidence-based medicine and philosophy of science perspective that citicoline should not be prescribed for acute ischemic stroke due to lack of efficacy and harm uncertainties.

Search publications: We searched in PubMed and Cochrane Library from 2020 until 30 October 2023. We, furthermore, used engineering machines Bing and Google Scholar to detect additional papers. Additionally, we also reviewed reference lists of the retrieved publications and review articles and searched the websites of the U. S. Food and Drug Administration (FDA) and European Medicines Agency (EMA).

Selection criteria: We included systematic reviews, meta-analyses, randomized clinical trials, clinical guidelines focused on acute ischemic stroke and comparing citicoline versus placebo or no intervention. We excluded narrative reviews, observational studies and ongoing trials.

Data collection and analysis: I identified only new randomized clinical trials and assessed the risk of bias in seven domains. The other eight trials were already included in the mentioned Cochrane review. The systematic reviews with or without meta-analyses were assessed using McMaster University guidelines. I estimated risk ratios (RRs) for that outcome. I measured statistical heterogeneity using the I^2 statistic. I conducted the analyses using the fixed effect model. I did not use the GRADE approach due to what is shown in a Cochrane review published in 2020 by Martí-Carvajal et al. I used the RevMan 5.4 software from Cochrane Collaboration to conduct the forest plot. I used a Trial Sequential Analysis with Copenhagen Trial Unit Software. I estimated a Bayes factor from the relative risk and 95% confidence interval.

Results: I identified only one new RCT (N = 99) reported mortality data and three clinical guidelines. I conducted a new meta-analysis with nine trials (N = 4461) having a high risk of bias and showing little to no difference in mortality between citicoline and placebo (17.1% vs 18.4%; RR 0.94, 95% CI 0.82 to 1.06; I^2 = 0%). The Bayes factor was 0.7, indicating weak Evidence for the null over the alternative hypothesis. Trial sequential analysis suggested sufficiency of Evidence for mortality. No guidelines recommend citicoline.

Conclusions: This essay reassessed citicoline for acute ischemic stroke after the 2020 Cochrane review. Adding a new RCT further supported the lack of mortality benefit with citicoline. The overall evidence quality could be better. Analyses

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using evidence-based medicine and philosophy of science approaches do not support prescribing citicoline due to a lack of efficacy substantiation and potential harms.

Keywords: citicoline; medical food; acute ischemic stroke; evidence-based medicine; philosophy of science.

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I. INTRODUCTION

In the ever-evolving field of medical science, the rigorous evaluation of treatment efficacy and safety is paramount. This essay delves into the complex and often contentious topic of citicoline, a medical food prescribed for ischemic stroke, whose effectiveness and safety profile have sparked considerable debate. Despite its widespread use, citicoline lacks definitive proof of efficacy, particularly in the context of all-cause mortality in stroke patients. This lack of conclusive evidence calls for a critical reexamination.

At the heart of this discussion lies a recent Cochrane review, which serves as a focal point for the analysis. While this essay does not replicate the depth of a Cochrane review, it aims to integrate key insights from this influential work with broader perspectives from Evidence-based Medicine and Philosophy of Science. In doing so, we navigate a multidimensional landscape that transcends the boundaries of conventional medical analysis, offering a more holistic view of the implications of citicoline use.

The primary objective here is not to provide specialized knowledge for experts in each respective field, but rather to present a global perspective, accessible to a wider audience. This approach underscores the importance of robust, high-quality evidence in guiding medical decisions and policies, especially when the stakes involve human health and wellbeing.

Through this essay, I will explore the current state of evidence regarding citicoline's efficacy and safety, weigh the arguments for and against its continued prescription, and propose a pathway forward, grounded in rigorous scientific inquiry and ethical consideration. In doing so, I aim to contribute to a more informed and balanced discourse on this critical healthcare issue.

1.1 Acute Ischemic Stroke: A Brief Overview

To accurately define ischemic stroke, it is essential to consider both clinical symptoms and evidence of infarction, providing a comprehensive understanding of the ischemia experienced by a patient. In cases of brief focal arterial ischemia lasting less than 24 hours without signs of infarction (either through pathology or imaging), the condition should be categorized as a transient ischemic attack (TIA).[1] Central nervous system infarction is defined as cell death in the brain, spinal cord, or retina due to ischemia, as evidenced by pathology, imaging, or other objective indicators of focal ischemic injury in a defined vascular distribution. Additionally, clinical evidence based on symptoms persisting for 24 hours or more, or until death, with other etiologies ruled out, is required.[1]

Acute ischemic stroke can be categorized into various types. The Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification system delineates ischemic stroke into five categories: 1) large-artery atherosclerosis, 2) cardioembolic events, 3) small vessel occlusion, 4) stroke of other determined etiology, and 5) stroke of undetermined etiology.[2]

Stroke remains a significant public health concern in the U.S., with an estimated 7 million Americans over 20 years of age having experienced a stroke. In 2016, there were nearly 800,000 new stroke incidents and 150,000 stroke-related deaths. The annual economic burden of stroke is substantial, costing the U.S. healthcare system approximately $45 billion.

However, there has been a decline in incidence since 1999, attributed to better control of

cardiovascular risk factors and advances in preventative treatments for arrhythmias. Despite this progress, more assertive prevention efforts, particularly focused on aging populations, are still imperative.[3-4]

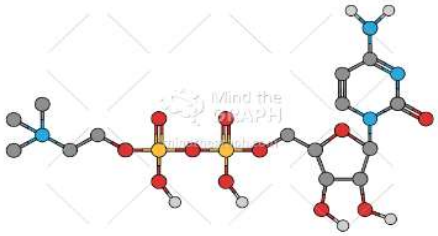
The economic toll of stroke is indeed significant, with an estimated annual cost of $45.5 billion during the 2014-2015 period.[4] This financial burden underscores the need for effective interventions and public health initiatives. In essence, while strides have been made in stroke management and prevention, the substantial impact of stroke in both human and economic terms highlights the urgency for continued research and effective public health strategies.

The clinical guidelines provide a comprehensive roadmap for the care of acute arterial ischemic stroke in adults, covering the continuum of care from the prehospital setting to hospital management, and emphasizing the initiation of secondary preventive measures within the first two weeks of the event. The integration of stroke systems, encompassing both prehospital and hospital settings, is a fundamental aspect of these recommendations.[5-7] In conclusion, the guidelines offer general advice based on the current evidence for physicians caring for adult patients with acute arterial ischemic stroke. However, it is important to acknowledge that existing data are often limited, highlighting the urgent need for ongoing research to further elucidate the treatment of this challenging condition. Saving lives and preventing debilitating outcomes will require not only the judicious application of current best practices but also an unwavering commitment to the pursuit of new knowledge.[5-7]

1.2 The Debate Surrounding Medical Foods and Supplements

In the vast spectrum of potential interventions, a particular class of treatment has stirred significant debate: medical foods and supplements. Citicoline, a medical food, has found itself at the center of this discourse (EFSA Panel 2013)[1].[8]

[1] EFSA denotes The European Food Safety Authority.



Made by Mind Graph for Arturo Martí-Carvajal (2023)

Figure 1: Citicoline. The structural formula of citicoline

Initially heralded for its purported neuroprotective properties, the promise of citicoline was that it might aid the brain's recovery post-stroke. However, as is often the case in the realm of medical science, early enthusiasm was met with subsequent scrutiny. Preliminary studies hinting at benefits were later juxtaposed against more rigorous evaluations that cast doubt over citicoline's efficacy. This dichotomy has not just sparked academic debates but has instigated broader contemplations about the ethics and implications of prescribing treatments shrouded in uncertainty.

According to the U. S. Food and Drug Administration's explicit guidance[9] and the European Union[10], citicoline is a medical food or a supplement, *not medication* or *drug*.

The FDA defines *medical foods* as "a food which is formulated to be consumed or administered enterally under the supervision of a physician and which is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognized scientific principles, are established by medical evaluation"[16]. Furthermore, it states that "Medical foods are not drugs and, therefore, are not subject to any regulatory requirements that specifically apply to drugs".[16]

The European Commission authorized citicoline as a novel food ingredient under food laws—neither categorized citicoline as a drug.[10] Citicoline's specifications approved uses, and labeling aligned with a food framework in major regulatory jurisdictions. The totality of evidence

indicates citicoline is considered a medical food or food ingredient, not a drug. Statements from the FDA and EU leave no room for interpretation and have definitively settled the issue: Citicoline is not a drug.

The World Health Organization's ATC/DDD system provides specifics about the classification and defined daily dose for citicoline.[11] ATC stands for Anatomical Therapeutic Chemical Classification System, which categorizes medicinal products based on the organ or system they act on and their therapeutic, pharmacological and chemical properties. DDD stands for Defined Daily Dose. It is the assumed average daily maintenance dose for a medicine's main indication in adults. The ATC/DDD system maintained by WHO categorizes citicoline under the code N06BX06. This signifies:

1. N06BX - Other psycho-stimulants and nootropics, a subgroup under the wider category of psycho-stimulants (N06B).
2. N06BX06 - The specific code for citicoline as an individual nootropic agent.

The Defined Daily Dose for citicoline is 0.8 grams, taken orally. This is the assumed average daily maintenance dose when used therapeutically. So in the ATC/DDD system, citicoline is classified as a psycho-stimulant nootropic. This provides a specification for global comparison and analysis.[11]

1.3 Relevance and Role of the Triadic Conceptual Graph

This essay employs a triadic conceptual framework, visually represented in the initial graph to navigate this intricate tapestry of evidence, philosophical considerations, and clinical realities. This illustration is not merely an aesthetic inclusion but serves as a navigational compass, elucidating the intersections of Evidence- Based Medicine (EBM), the Philosophy of Science, and citicoline. By providing this snapshot at the outset, readers are equipped with a mental map, guiding them through the nuanced discussions that follow.

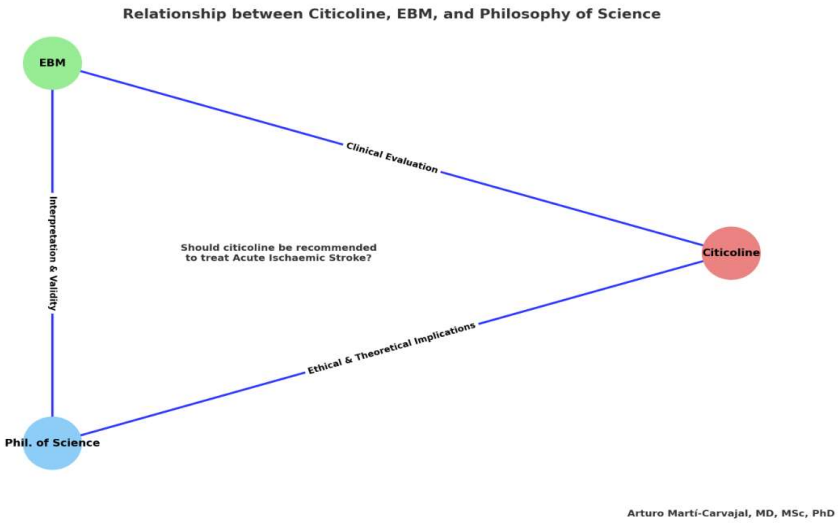


Figure 2: Relationship between Evidence-based Medicine (EBM), Citicoline and Philosophy of Science.

The Triadic Conceptual Graph: An Essence

At the crux of this analytical piece, we navigate through a triadic conceptual graph, intricately woven to explore the intricate dynamics between Evidence-Based Medicine (EBM), the Philosophy of Science, and citicoline in acute ischemic stroke scenarios. This graph transcends mere aesthetic value, encapsulating the foundational beliefs of the essay and visually mapping out the complex interrelations among these pivotal domains.

1.3.1. The Bedrock and Cornerstone: Evidence-Based Medicine (EBM)

In our conceptual triad, the 'EBM' vertex stands resilient, serving both as the bedrock and cornerstone of our exploration. It epitomizes the stringent methodologies and guiding principles imperative for medical decisions rooted in systematically evaluated and critically reviewed evidence. This vertex draws strength from the pioneering works of Archibald 'Archie' L. Cochrane[12] and David L. Sackett[13-14], who fervently advocated for melding clinical acumen with the highest caliber of research evidence. Positioned at the triad's foundation, EBM stands as the moral imperative for clinicians, beckoning them to harmonize their individual expertise with the paramount external evidence in their medical deliberations. EBM's guiding light directs us toward systematic reviews, converging the apexes of clinical research to inform medical practice and elevate patient care to its zenith.

A key philosophical principle underpinning EBM is Ockham's razor[15], which favors the simplest explanation supported by the evidence. This aligns with EBM's hierarchical prioritization of evidence quality, with randomized trials at the top. Ockham's razor reinforces EBM's wariness of anecdotal data or speculative pathophysiology as sole justification for treatments. Applying this to citicoline, the lack of robust efficacy and safety evidence in ischemic stroke points to discontinuing its prescription as the simplest conclusion, rather than hypothesizing unsubstantiated benefits and risks. As Simon and Rios[16] stated, principles like Ockham's razor are crucial for evidence-based practice to avoid perpetuating unsupported interventions. Integrating such philosophy expands EBM's methodological rigor.

1.3.2 The Prism: Philosophy of Science

On the opposing end, the 'Philosophy of Science' vertex unravels the profound epistemological layers of scientific investigation. Illuminated by intellectual giants such as Karl Popper[17] and the Vienna Circle[18], this vertex scrutinizes the methodologies, ethical considerations, and foundational beliefs that sculpt our perception of scientific truth. The left vertex casts a philosophical gaze upon science, dissecting the methodologies, ethics, logic, and epistemology that constitute scientific knowledge. Figures like Sir Karl Popper, Michel Foucault and Sir Peter Medawar have intricately woven the fabric of understanding, dictating how scientific theories are birthed, validated, and embraced. Through the philosophy of science's lens, we critically appraise the underlying biases, contexts, and assumptions permeating biomedical research.

1.3.3 The Exemplar: Citicoline

The 'Citicoline' vertex, positioned at the triad's pinnacle, symbolizes the medical food attributed with neuroprotective potentials, serving as the essay's focal case study.[16] Citicoline, believed to offer neuroprotection post-acute ischemic stroke, stands as the concrete example scrutinized through the EBM and Philosophy of Science frameworks. In this crucible of medicine, citicoline manifests as a nexus where ethical considerations, scientific validation, and philosophical introspections converge.

1.3.4 The Unifying Query

Interlinking these domains is the pivotal question: Is citicoline a viable prescription for acute ischemic stroke patients? This inquiry, deeply entrenched in efficacy, safety, and ethical considerations, propels our exploration forward.

The triadic synergy of EBM, philosophical inquiry, and the citicoline discourse amalgamates to scrutinize this central dilemma.

In essence, the triadic graph serves as a conceptual compass, guiding us through the pivotal realms of evidence-based medicine, scientific philosophy, and the nuanced clinical debate surrounding citicoline. Both visually and narratively, the graph sheds light on the synergistic integration of these elements, informing the pivotal question regarding citicoline's role in clinical practice. At the triangle's heart, the urgent research inquiry arises, tying together the vertices and encouraging a holistic evaluation of citicoline, transcending empirical evidence and delving into philosophical depths. This graph, therefore, encapsulates the

essay's odyssey, a quest through evidence, philosophical inquiry, and clinical realities, in pursuit of answers with far-reaching implications for patient care and societal well-being.

II. RESEARCH QUESTION

Why citicoline (a medical food) should not be prescribed to treat people with acute ischemic stroke: The certainty of the evidence?

The journey of scientific discovery often mirrors the profound depths of linguistic etymology, weaving a rich tapestry of history, meaning and potential. The term "etiology" epitomizes this parallel. Derived from the Greek words "aitia" (cause) and "logos" word proposed by Heraclitus of Ephesus in 540 BCE (study, reason), etiology encapsulates the relentless quest to comprehend causation and the origins of phenomena.

In medicine, and specifically in investigating acute ischemic stroke, unraveling etiology acquires crucial significance. Like skilled linguists deciphering semantic evolution, physicians probe complex pathophysiology, seeking reasons and definitive evidence to guide therapeutic choices. At this crossroads, armed with robust research methodology and an unwavering spirit of inquiry, the scientific community strives to illuminate the obscured etiology of disease and construct the most apt interventions.

In this spirit, we explore a compound that holds promise against the ravages of acute ischemic stroke – citicoline. The scientific world anticipates robust etiological understanding that could elevate citicoline from possibility to proven prescription. Nevertheless, assertions require rigorous scrutiny; potential must transition to evidence-based efficacy before infiltrating clinical practice.

With ethical principles as our guideposts, we embark on an odyssey through clinical trials and systematic insights, seeking the elusive etiology that transforms therapeutic speculation into a confident recommendation. Like language detectives united by curiosity, we persevere until science speaks clearly.

This spirit of scientific rigor and objective analysis, guided by principles of beneficence and nonmaleficence (First, do not harm!) leads us to investigate the central research question systematically: Should citicoline be prescribed for individuals with acute ischemic stroke? The answer awaits at the journey's end.

III. OBJECTIVE

Our primary aim in this essay is to conduct a rigorous analysis of citicoline's clinical effectiveness and examine the ethical dimensions surrounding its use for acute ischemic stroke. We approach this evaluation through the lenses of Evidence-Based Medicine and Philosophy of Science, incorporating insights from esteemed thinkers like Michel Foucault, Sir Karl Popper, and Sir Peter Medawar. While citicoline's intricate pharmacodynamics and pharmacokinetics warrant exploration, dissecting these mechanisms in detail extends beyond the scope of this current work. We focus on synthesizing the highest quality clinical evidence regarding citicoline's efficacy and safety for acute ischemic stroke and weighing the implications of prescribing this medical food through an ethical lens. By integrating principles of clinical epidemiology and scientific philosophy, we seek to reach judicious conclusions rooted in both empirical data and moral reasoning, elucidating not just what can be done but what should be done to benefit patients in keeping with ethical medical practice.

IV. METHODOLOGY: NAVIGATING THE SEA OF EVIDENCE

In scientific inquiry, methodology is the beacon that guides our exploration, ensuring that the journey is rigorous, systematic, and unbiased. Our methodology in this essay is rooted in a multidimensional approach, striving to balance empirical evidence, critical analysis, and best practices in research evaluation.

4.1 Sources of Data and Document Evaluation

Our starting point was a meticulous selection and evaluation of data sources. The vast expanse of biomedical literature, teeming with studies,

reviews, and reports, necessitates a discerning eye. We focused on randomized clinical trials (RCTs) that emerged post-2020, ensuring their alignment with the criteria established by the Cochrane review on citicoline[19]. For this essay, each document underwent a thorough vetting process, evaluating its relevance, credibility, and contribution to the overarching research question.

We searched in PubMed using the clinical query approaches recommended by that database and Cochrane Central Register of Controlled Trials (CENTRAL). Furthermore, we searched in Google Scholar and the search engine Bing from Microsoft. The search was limited to 2020 to October 15, 2023. We also searched Clinical Guidelines from scientific organizations for retrieving potential recommendations about the citicoline's use in people with acute ischemic stroke.

In our rigorous journey to uncover the intricacies of citicoline's role in acute ischemic stroke, we have meticulously chosen our sources of evidence, adhering to the highest standards of scientific inquiry. A pivotal decision in our methodology was to exclude either narrative reviews or ongoing trials from our pool of resources. This decision was rooted in our commitment to uphold the principles of Evidence-Based Medicine (EBM) and ensure the robustness of our conclusions.

Narrative reviews, while informative and often insightful, do not adhere to the systematic and transparent methods characteristic of systematic reviews and randomized controlled trials. They are typically authored based on the personal expertise and interpretative skills of the authors, and they may not follow a predefined protocol for literature search and selection. This subjective nature can introduce bias, as the selection of studies and the interpretation of results may be influenced by the authors' perspectives and preferences.

In contrast, systematic reviews and RCTs offer a more objective and reproducible approach to evidence synthesis. They follow strict protocols for literature search, study selection, and data extraction, minimizing the risk of bias and ensuring that the conclusions drawn are based on a comprehensive and balanced view of the available evidence. By focusing on these sources, we aim to provide a more reliable and unbiased assessment of citicoline's efficacy in acute ischemic stroke.

Our commitment to EBM principles guided our decision to exclude narrative reviews, as we sought to base our conclusions on the highest quality of evidence available. This approach ensures that our findings are grounded in robust scientific data, providing a trustworthy and valid contribution to the ongoing discourse on citicoline and acute ischemic stroke. This focused justification clarifies the rationale behind excluding narrative reviews, emphasizing the commitment to EBM principles and the pursuit of unbiased and reliable evidence.

We followed the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta- Analyses) flow diagram in this manuscript to demonstrate the author's firm commitment to the highest reporting standards for systematic reviews.[20] However, we have clearly stated that this manuscript is not an update of the Cochrane review mentioned earlier.

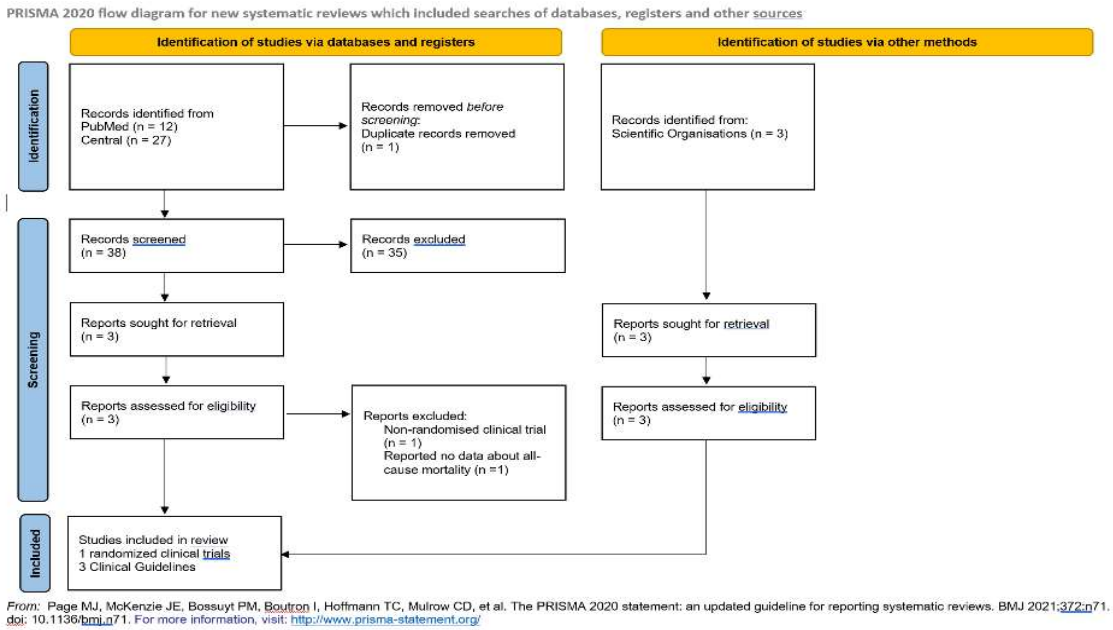


Figure 3: Deciphering the Tapestry of Research: A Comprehensive PRISMA-Guided Systematic Review on Citicoline in Acute Ischemic Stroke

PRISMA is an evidence-based guideline that consists of a checklist and flow diagram to improve the reporting of systematic reviews and meta-analyses. By following PRISMA guidelines, the author provides complete transparency about their rigorous review methodology, thus increasing confidence in their findings.

Specifically, the diagram details every step of the study selection process, from the comprehensive search across multiple sources through the careful application of eligibility criteria to the final decision of which studies to include in the synthesis. Readers can follow the path traced by the authors in their systematic pursuit of the best available evidence. The adherence to PRISMA, an established reporting guideline, sends an unambiguous signal: this is no rushed or sloppy work. Instead, it is a rigorous, meticulous, systematic review worthy of the highest consideration from the scientific community. The findings presented deserve to be taken seriously, given the painstaking process that produced them.

In conclusion, the presence of a PRISMA flow diagram establishes the high methodological standards of this review and reaffirms the commitment to transparency and scientific excellence. Its inclusion warrants confidence and validation of the critical findings reported herein.

4.2. Application of Cochrane Collaboration Recommendations

The Cochrane Collaboration stands as a gold standard in the world of systematic reviews, renowned for its rigorous methodologies and dedication to evidence-based medicine. In our endeavor, we leaned heavily on the Cochrane Collaboration's recommendations[20], especially in synthesizing and interpreting the data from various RCTs. Their guidelines offered a structured framework, enabling us to assess the quality of evidence, minimize biases, and derive meaningful conclusions about citicoline's efficacy in treating acute ischemic stroke. We conducted a new meta-analysis including a new RCT published in 2022.[21]. The meta-analysis was by subgroup due to the new RCT assessed citicoline administered immediately after recanalization therapy for acute ischemic stroke.[21] No trial included Cochrane review[19] had been used Agarwal's approach. To assess the imprecision, we followed the recommendations from GRADE group.[22] We limited this essay to the main primary outcome of the Cochrane review[19]: all-cause mortality. Full details about the other outcomes are available in Martí-Carvajal, et al.[19]

4.3 Critical Analysis of Biomedical Literature

A systematic review requires more than just a rigorous process - it demands critical analysis. We looked beyond the surface data, evaluating each trial and report as a product of its context, biases, and assumptions. In the construction of the evidence in this essay, our goal was not merely aggregating empirical findings, but gaining a complete picture of the evidence - both the tangible results and the wider scientific narratives that shape our comprehension. This comprehensive scrutiny and skepticism allow moving closer towards an accurate understanding of citicoline for stroke, though absolute truth likely remains elusive. A thoughtful systematic review illuminates both what we know and how we know it.

4.4 Trial Sequential Analysis

Our trial sequential analysis methodology aligns with the approach explained in detail by Martí-Carvajal, et al.[19]. In summary, adding the new RCT, we recalculated the required information size, or minimum sample size needed, accounting for heterogeneity and using a 20% relative risk reduction, 80% power, 5% alpha, and diversity adjustment.

Based on the required information size, I constructed trial sequential monitoring boundaries to assess whether the cumulative evidence definitively proves or disproves the hypothesized treatment effect before reaching the required sample size. Crossing the boundary indicates further trials are likely unnecessary, while not reaching the boundary signals additional trials are still needed. I applied trial sequential analysis software to evaluate if the cumulative meta-analysis crossed the futility boundary.

Per my typical approach, I only conducted trial sequential analysis for the primary outcome of all-cause mortality. This aligns with the methodology I have followed and described extensively in prior publications to optimize type I and II error control and objectively evaluate futility or conclusiveness of cumulative randomized evidence. I believe using trial sequential analysis provides a scientifically rigorous way to determine the need for additional trials. I conducted TSA using software from the Copenhagen Trial Unit.[23]

I reconducted the new TSA because we found one trial reporting all-cause mortality.[21] It was an ongoing trial when the Cochrane review on citicoline was published in 2020.[19]

In sum, our methodology was a fusion of systematic processes, critical thinking, and philosophical inquiry, all converging to shed light on the central question of Citicoline's role in acute ischemic stroke management.

V. THE ART OF SOLUBLE AND NAVIGATING THE MYSTERY OF CITICOLINE

In the vast landscape of medical science, there exists a dynamic interplay between persistent queries and emerging answers. Sir Peter Medawar, in "The Art of the Soluble,"[24] eloquently delves into this dance of scientific inquiry, suggesting that the true mettle of science isn't just in the solutions it offers, but in its capacity to discern which problems are ripe for resolution.

5.1 Medawar's Meditations in the Context of Citicoline

Medawar championed the idea that the brilliance of a scientist is reflected not just in the answers they uncover, but in the questions, they dare to ask.[24] Applying this philosophy to the debate surrounding citicoline, one might posit: is the question of its efficacy in treating acute ischemic stroke a "soluble" problem? The journey to ascertain whether citicoline should be prescribed is not merely a quest for evidence but an exploration of whether the mystery surrounding its use is one that can be unraveled through rigorous scientific inquiry. Peter Medawar's wisdom highlights focusing on soluble questions - those with accessible answers that advance understanding. Though intellectually appealing, insoluble problems risk stagnation. Progress lies in channeling curiosity toward questions matched to current capabilities. The soluble may reveal

provisional truths, even if initially wrong. Partial answers expose the substratum where greater truth rests. In research, asking pertinent questions ripe for attainable solutions, given present limitations, allows science to expand its edifice brick by brick. Medawar advocates pursuing the soluble, not to sidestep difficulty, but for tractable progress. His razor-sharp insight cuts to the core - growth of knowledge hinges on properly framing the knowable.

5.2 The Hypothesis-Driven Exploration of Citicoline's Efficacy

At the heart of this quest lies the hypothesis. Beyond being a tentative assertion, it serves as the compass directing our exploration. In the context of citicoline, the guiding hypothesis challenges us to assess its therapeutic potential for acute ischemic stroke. Each clinical trial, every patient's experience, and all observational data become integral components in either affirming or refuting this hypothesis. The world of evidence-based research, then, isn't just about amassing data but about critically evaluating this data against our guiding question.

In essence, Medawar's reflections on the art of problem-solving in science serve as a philosophical backdrop to our exploration of citicoline. It underscores the journey as one of discernment—identifying the right questions, seeking solvable problems, and navigating the maze of evidence to arrive at a conclusion that resonates with both scientific rigor and clinical relevance.

VI. THE SCAFFOLD OF SCIENTIFIC TRUST: DECIPHERING THE HIERARCHY OF EVIDENCE

In the grand tapestry of medical science, not all evidence is woven with the same thread. The strength, consistency, and reliability of scientific findings vary, necessitating a structured approach to discern the weight of different types of evidence. This is where the concept of the 'Hierarchy of Evidence' comes to the fore, acting as a beacon for clinicians and researchers navigating the vast seas of medical literature.[25]

6.1 The Essence of Evidence-Based Medicine (EBM)

Evidence-Based Medicine, at its core, is a commitment—a vow to ensure that clinical decisions are anchored in the most robust and relevant evidence available. But how does one determine what constitutes 'robust' evidence? EBM doesn't just champion the use of evidence; it emphasizes the *quality* of that evidence. The Hierarchy of Evidence[25], in essence, is a tool that helps segregate studies based on their methodological rigor and susceptibility to bias. From case reports at the base to randomized controlled trials higher up, each rung of this ladder represents a different level of trust-worthiness.

6.2 The Cochrane Review: A Gold Standard in Medical Research

Perched at the zenith of this hierarchical pyramid is the systematic review, and among them, the Cochrane systematic review is often considered the gold standard.[20] Why? Because it embodies the very principles EBM holds dear: comprehensiveness, rigor, transparency, and a commitment to minimizing bias. Cochrane reviews synthesize the best available evidence on a given topic, subjecting individual studies to meticulous scrutiny and pooling data to provide a consolidated view. When it comes to understanding the role of citicoline in acute ischemic stroke, the Cochrane review serves as a lighthouse, illuminating the path with its rigorous analysis and evidence synthesis.

VII. DIVING INTO THE COCHRANE SYSTEMATIC REVIEW

The domain of medical inquiry is immensely broad, weaving a complex tapestry of studies, assertions, and counterarguments. Within this intricate expanse, systematic reviews stand as pivotal navigational beacons, with those orchestrated by the esteemed Cochrane Collaboration[26] holding a particularly luminous position. These comprehensive analyses act as compasses, steering clinicians, researchers, and policymakers through the intricate maze toward conclusions grounded in robust evidence.

In this critical juncture of our exploration, we immerse ourselves in an in-depth examination of the Cochrane review that scrutinizes the role of citicoline in the management of acute ischemic stroke.[19] This review, distinguished by its meticulous methodology and commitment to impartiality, serves as a cornerstone in our understanding, offering a well-rounded and discerning perspective on this pivotal medical inquiry.

We stand at the precipice, ready to unravel the layers, navigate the complexities, and derive clarity from the wealth of accumulated knowledge. It is through this lens of rigorous evaluation and keen insight that we endeavor to shed light on the intricate interplay of factors surrounding the use of citicoline, guiding our journey through the vast expanse of medical knowledge.

7.1 The Cochrane Gold Standard

The Cochrane Collaboration's stringent methodological criteria and commitment to minimizing bias ensure its systematic reviews represent the highest quality evidence.[20]

By meticulously combining individual studies and extracting meaningful truths, Cochrane reviews serve as a compass to guide understanding of healthcare interventions. In 2020, Martí-Carvajal et al. published a Cochrane review[19] assessing the clinical benefits and harms of citicoline for treating acute ischemic stroke patients. This review was essential to evaluate citicoline amid conflicting prior evidence. While some studies suggested benefits, others showed no significant improvements versus placebo or controls.

Furthermore, previous meta-analyses had limitations, including lack of Trial Sequential Analysis and suboptimal effect measures. With many industry-funded trials, independent assessment was needed. Despite inconclusive evidence, some countries still prescribed citicoline. By adhering to rigorous Cochrane methods, this review aimed to conclusively determine if citicoline provided meaningful clinical benefits and harms compared to other acute ischemic stroke treatments. The conclusion of the authors was (verbatim): *This review assessed the clinical benefits and harms of citicoline compared with placebo or any other standard treatment for people with acute ischemic stroke. The findings of the review suggest there may be little to no difference between citicoline and its controls regarding all-cause mortality, disability or dependence in daily activities, functional recovery, neurological function and severe adverse events, based on low-certainty evidence. None of the included trials assessed quality of life and the safety profile of citicoline remains unknown. The available evidence is of low quality due to either limitations in the design or execution of the trials.* For full details, readers can refer to Martí-Carvajal, et al 2020.[19]

7.2 Unraveling the Findings

At the intersection of hope and skepticism, where anecdotal experiences meet rigorous scientific scrutiny, the Cochrane review on citicoline offers a synthesized perspective. Drawing from a multitude of randomized controlled trials, it provides a panoramic view of citicoline's impact on acute ischemic stroke. The results, encapsulated visually in the forest plot, offer a clear picture of the effect sizes from individual studies and their combined influence.

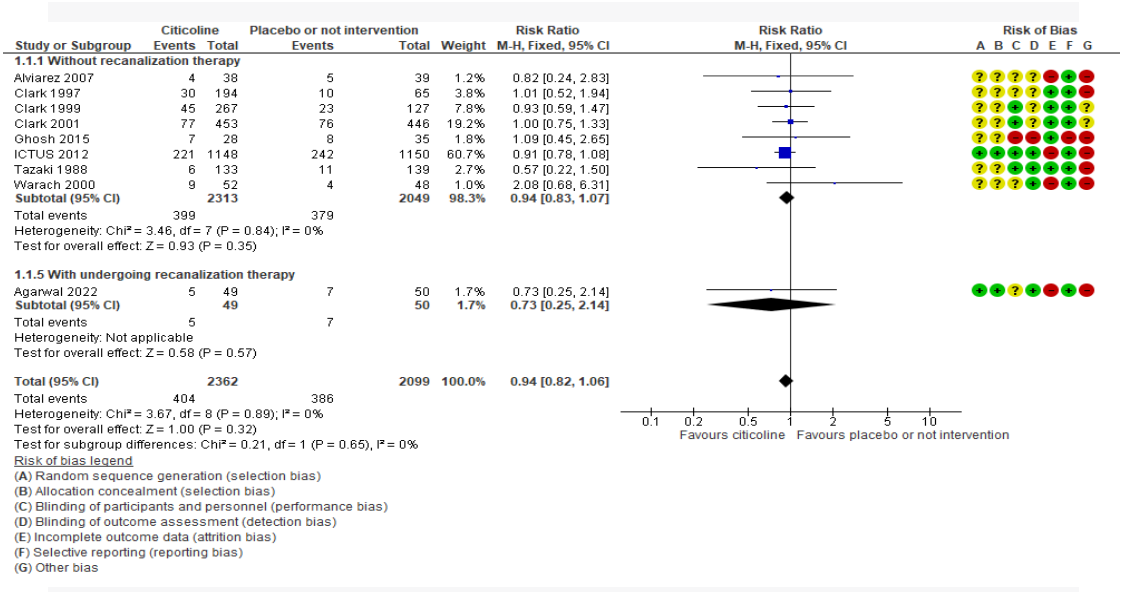


Figure 4: Forest Plot Depicting the Effect of Citicoline on All-Cause Mortality in People With Acute Ischemic Stroke Based on Subgroup Analysis

The forest plot displays the results of nine individual studies categorized into two subgroups. The horizontal lines represent 95% confidence intervals for each study's effect estimate. The squares denote the risk ratio point estimates, with the size of the square proportional to the study's weight in the meta-analysis. The vertical line indicates the null effect of relative risk = 1. The diamonds represent the pooled effect estimates from a meta-analysis using a fixed-effect model.

The first subgroup includes eight trials without recanalization therapy. The pooled risk ratio is 0.94 (95% CI 0.83 to 1.07), with low heterogeneity (I^2 = 0%). The total sample size was 4362 patients, representing 13.65% of the optimal information size (4362/31900) required to detect a significant treatment effect conclusively. The 95% CI includes the possibility of no mortality benefit.

The second subgroup comprises one trial involving patients undergoing recanalization therapy.[21] This trial showed a risk ratio 0.73 (95% CI 0.25 to 2.14). The sample size of 99 patients is 4.78% of the optimal information size (99/2070). The wide 95 % CI includes the possibility of no treatment effect.

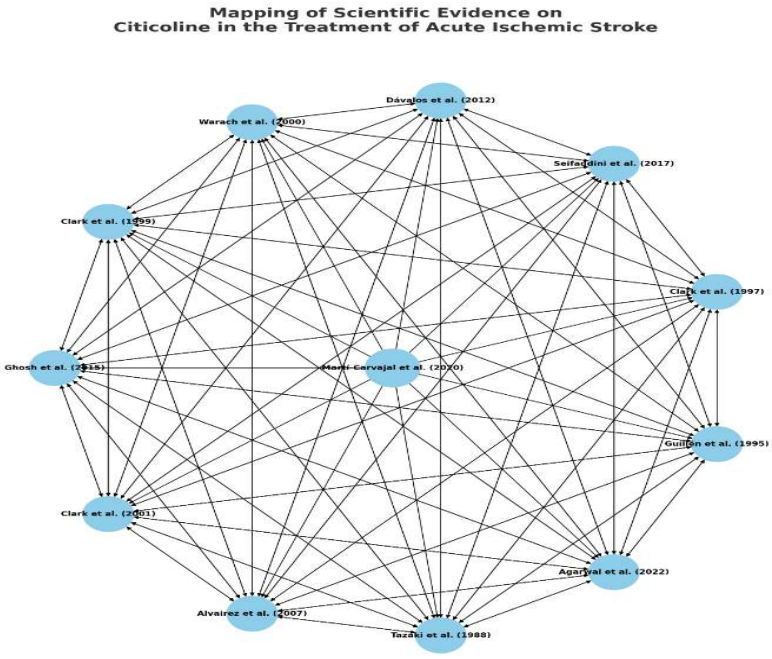
The forest plot encompasses nine trials with 4461 participants. The overall meta-analysis risk ratio is 0.94 (95% CI 0.86 to 1.06, I^2: 0%), suggesting no significant mortality reduction with citicoline compared to placebo/no intervention. The total sample represents 14% of the optimal information size required, indicating imprecision. Furthermore, the 95% CI includes the possibility of no benefit. The test for subgroup differences was P = 0.65 and I^2: 0%. Bayes factor was 0.93, using the RR and its 95% CI of the meta-analysis. It indicates that the data is inconclusive - the evidence does not favor either the null hypothesis or the alternative hypothesis. The Bayes factor of 0.93 shows that the data do not provide enough evidence to conclude either way about the effect. More data would be needed to strengthen the evidence in favor of the null or alternative hypothesis.

The Bayesian analysis may be limited by potentially inappropriate prior and assumptions. With limited data, results can be sensitive to outliers. The analysis relied solely on the meta-analytic data without considering the broader theoretical and empirical landscape. While the Bayes factor is tentatively aligned with the lack of efficacy, over-interpreting statistical analyses without grounding in the literature can lead to spurious conclusions.

Based on the current evidence from this meta-analysis, there is no convincing mortality

benefit for citicoline compared to placebo/no intervention in patients with acute ischemic stroke. The totality of data needs to be revised to demonstrate any treatment effect due to suboptimal sample size and imprecision. Additionally, the included trials have a high risk of bias, severely limiting the reliability of the results. There is no significant statistical heterogeneity.

In brief, the evidence firmly indicates citicoline provides no mortality advantage over placebo or standard care in acute ischemic stroke people. The meta-analysis results are decisively null regarding mortality reduction. The judgment of certainty is very low due to the imprecision and high risk of bias. Currently, the evidence convincingly fails to support using citicoline, a medical food, specifically to reduce mortality in this population.



Arturo Martí-Carvajal (2023)

Figure 5: Mapping of Scientific Evidence on Citicoline in the Treatment of Acute Ischemic Stroke

The graph unfolds as a meticulously constructed network of randomized clinical trials, each delving into the intricacies of citicoline's role in treating acute ischemic stroke. In this visual representation, individual studies are denoted as nodes, while the edges draw lines of potential relationships and scholarly dialogues between them.

In scientific inquiry, each study is a beacon of knowledge, yet it does not stand in isolation. Much like the famous poet John Donne (1572–1631) once eloquently expressed, 'No man is an island,' this network of trials epitomizes the interconnected nature of scientific pursuit. The graph emphasizes that each piece of research, each node in this network, contributes to a collective understanding, building bridges of knowledge and insight across the expanse of medical literature. In essence, this visual narrative serves not just as a map of existing research but also as a reminder that in pursuing scientific truth, each study is a vital part of the greater whole, interconnected, and indispensable.

It remembers the critical thinking from Sir Peter Medawar, as mentioned. Specifically, Medawar's emphasis on focusing scientific inquiry on "soluble" questions which can advance understanding, rather than getting stuck on insoluble problems, is tremendously valuable advice.

As Medawar elegantly states: "There are questions which science cannot answer, and it is no good

beating one's head against these questions. What matters is to learn to discriminate between soluble questions with real and accessible answers, and questions that are beyond solution for the time being." This highlights the wisdom of channeling curiosity toward questions matched to current capabilities, while appreciating present limitations. Progress lies in pursuit of the soluble.

Medawar also astutely notes that even incorrect answers can unveil fragments of truth and "expose the substratum on which truth rests." Science expands its edifice brick by brick through this spirit of imaginative, yet grounded, inquiry. These philosophical principles remain highly pertinent, both broadly to research methodology and specifically to exploring controversial areas like the citicoline debate. Focusing on judiciously framed, tractable questions allows the gradual accretion of knowledge.

Martí-Carvajal, et al.[19], standing as the root of this intricate network, occupies a central position, symbolizing its comprehensive nature and its pivotal role in analyzing multiple studies on the topic. As a Cochrane review, it holds a high standard of evidence, serving as a linchpin in the network and a reliable guide for clinical decision-making.

The other nodes, representing various randomized clinical trials, showcase the diverse investigations conducted over the years, each contributing unique insights into the effects of citicoline across different settings and populations. These studies are not isolated islands of knowledge; they are interconnected parts of a larger conversation, contributing to and influenced by the collective understanding synthesized in the Cochrane review.

The edges, or connections, represent the relationships between the studies, highlighting how each is interconnected, complementing, contrasting, and contributing to the broader research landscape on citicoline in acute ischemic stroke.

The node sizes visually represent the relative importance or weight of the studies, with the Cochrane review standing out due to its comprehensive and systematic nature, serving as a testament to the power of collective inquiry and the crucial role of systematic reviews in navigating the complex seas of medical research.

7.3 Beyond Numbers - Implications for Practice

While the data and statistics form the backbone of the review, their actual value lies in their clinical implications. How does the evidence from the Cochrane review translate to bedside decisions? What does it mean for a patient with acute ischemic stroke awaiting treatment decisions? These are the pressing questions this section seeks to answer, marrying numbers with narratives and research with real-world ramifications. It is the essence of this essay: First, do not harm! This paragraph brings to Sir Karl Popper's thoughts[27].

Evidence-based medicine, though accused of dogmatism, strongly aligns with Karl Popper's falsification principles. At its core, robustly testing medical claims against empirical scrutiny. This spirit of critical inquiry, of tentatively gleaning truth through refutation, permeates EBM's essence. Yet clinical practice demands more than an abstract search for statistical certainty. It requires a profoundly humanistic approach centered on understanding patients' values and goals. Formulating the initial question reveals this truth. It necessitates appraising internal expertise and anticipating real-world applicability. But crucially, it involves an intimate exploration of patients' preferences to establish mutually agreeable objectives. Here, we grapple with ethically complex decisions affecting others' wellbeing. Mastering empathy, counseling, and narrative medicine becomes vital to properly frame the clinical question. We must see our patients not as data points or outcomes, but as people hoping to be heard, understood, and empowered. Evidence-based medicine, robustly practiced, is this weaving together of science and compassion. The meticulous examination of research evidence requires pairing with the humanistic art of opening dialogue. In this full embodiment of EBM, we stay grounded in the Popperian principles of critical thinking while elevating care to an act of moral agency and human connection.[27]

7.4 Trial Sequential Analysis (TSA) - The Final Seal

Supplementing the systematic review's findings, the TSA offers another layer of evidence.[28] By visually depicting the trajectory of cumulative data, it can demonstrate whether more trials on citicoline are warranted or if the evidence has reached a point of futility. In the case of citicoline, the TSA provides compelling insights into the medical food's future research needs.

To determine whether additional trials are needed to evaluate the effect of citicoline on all-cause mortality conclusively, we conducted a trial sequential analysis incorporating the cumulative data from the trials included in the meta-analysis forest plot (Figure 4).

Figure 6 displays the trial sequential analysis results for all-cause mortality, including all trials in the prior meta-analysis.

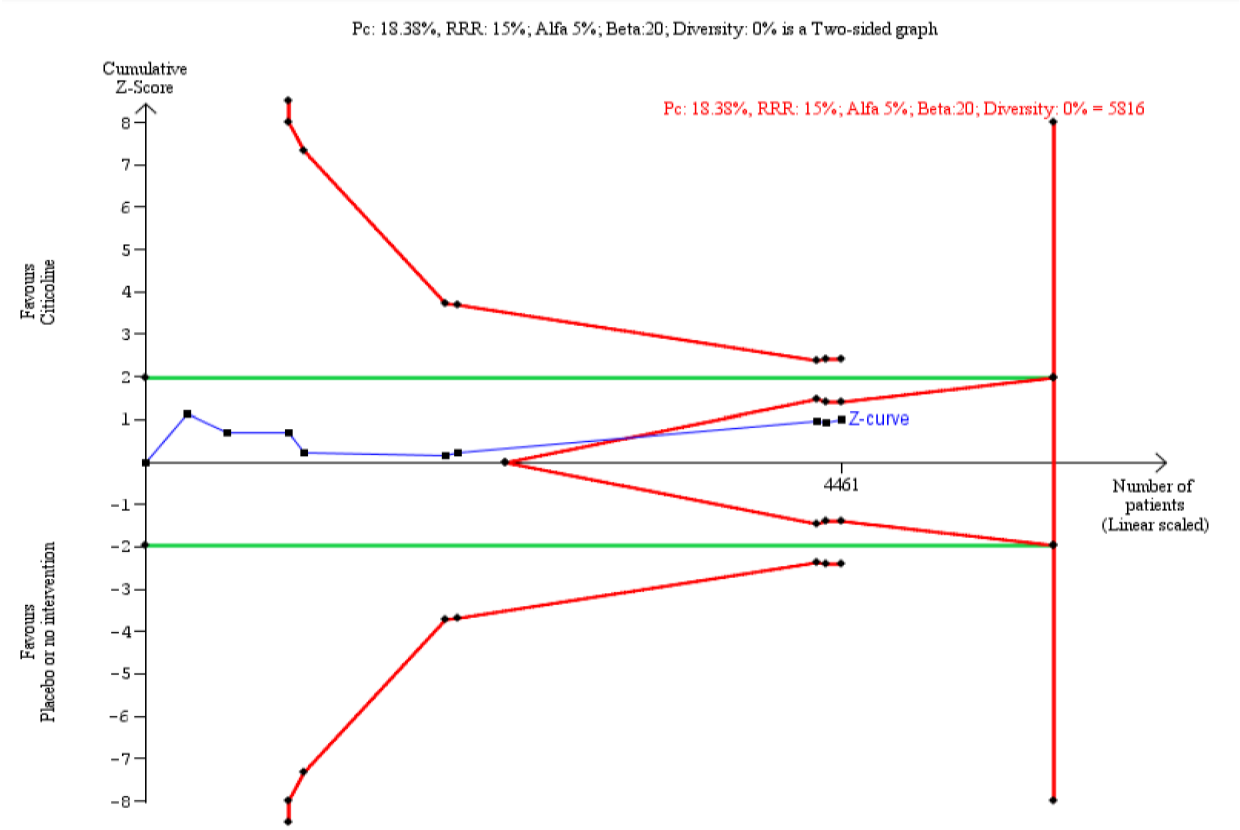


Figure 6: Trial Sequential Analysis for citicoline versus placebo or no intervention on all-cause mortality

The trial sequential analysis reveals the diversity-adjusted required information size to conclusively detect or reject a 15% relative risk reduction in all-cause mortality was calculated to be approximately 5816 patients, based on the proportion of events in the control group and specified α and β levels. The cumulative Z-curve crossed the futility boundary after just seven trials, indicating firm evidence that further trials are unlikely to alter the conclusion of no significant mortality difference between citicoline and control.

The totality of current data provides decisive evidence that citicoline does not confer a mortality benefit compared to placebo or no intervention in this population. Achieving the required information size of 5816 patients is unnecessary, as futility has already been demonstrated. No single trial individually reached

statistical significance for mortality. There is absolutely no indication additional randomized controlled trials on this outcome are warranted, as the cumulative results appear resolute that citicoline does not significantly reduce all-cause mortality risk compared to placebo or no comparison. The trial sequential analysis provides compelling evidence that further investigation of this particular outcome is futile.

7.5 Concluding thoughts

Armed with the rigorous analysis of the Cochrane review and the visual clarity of the forest plot and TSA, this section provides readers with a comprehensive understanding of citicoline's role in acute ischemic stroke. It underscores the importance of evidence-based practice, urging clinicians to base their decisions on the best available evidence, even when it challenges long-held beliefs or popular narratives. There is a need to address that citicoline is not a medication but a medical food or supplement.

VIII. PROBING THE CITICOLINE CONTROVERSY: A DISCOURSE ANALYSIS THROUGH POPPER, MEDAWAR AND FOUCAULT

This section directs us back to Figure #1: the graph illustrating the intricate relationship between the Philosophy of Science, Evidence-Based Medicine, and Citicoline. Let us delve deeper into this interconnectedness.

The intersection of Evidence-Based Medicine (EBM), Philosophy of Science, and the specific topic of Citicoline in the scope of acute ischemic stroke, as visualized in the graph, is a critical juncture. The narrative we have constructed around Popper's Medawar's and Foucault's philosophies concerning EBM and the citicoline debate serves as a central pillar of the essay. This confluence of ideas at the vertex reinforces the core question and themes of this essay, emphasizing the intertwined nature of scientific inquiry, philosophical understanding, and medical practice. The graph visually symbolizes this connection, and the narrative explanation delves deeper into its essence and significance.

8.1 Bridging the Divide: A Comparative Evaluation of Current Evidence on Citicoline for Acute Ischemic Stroke

Despite differences in scope and methods compared to this essay, the systematic review by Sagaro et al. aligned with our essay, concluding that citicoline does not significantly improve outcomes for patients with acute ischemic or hemorrhagic stroke.[20]

Given the lack of proven benefit, philosophers like Immanuel Kant might logically question the rationale for continuing to promote citicoline for stroke treatment. Ischemic stroke has high mortality and disability, so proposed therapies require rigorous support (Gonzales 2022)[30]. While preclinical citicoline studies seem promising, benefits have not translated in phase 3 trials[19]. More research is required to elucidate mechanisms in humans and determine if citicoline provides tangible improvements in stroke patients.

According to the TSA, no more RCTs are needed for citicoline in people with acute ischemic stroke (Data are not shown in this essay). In summary, this assessment raises valid questions about the justification for citicoline use in stroke care, given inconclusive clinical trial findings to date.

8.2. Philosophy of Science and Evidence-based Medicine

8.1.1 Bridging two Domains

The discipline of medicine, while rooted in the empirical, is also profoundly entangled with philosophy. The practice of medicine is not merely the application of biological knowledge but also engages with more profound questions about the nature of evidence, the structure of scientific inquiry, and the ethics of clinical practice. This intersection of the philosophy of science and evidence-based medicine offers a rich tapestry of ideas that can inform and refine the practice of medicine.

8.1.2 Popperian Falsification and the Citicoline Debate

Sir Karl Popper, one of the most influential philosophers of science, posited that scientific theories can never be proven, only disproven. This principle of falsification becomes particularly pertinent in the debate around citicoline. If the evidence from randomized controlled trials and systematic reviews, such as the Cochrane review, consistently fails to support the efficacy of citicoline in acute ischemic stroke, then the Popperian approach would argue against its use. The essence of Popper's philosophy urges us to be skeptics, to challenge prevailing notions, and to let evidence guide our beliefs and practices.[14]

8.1.3 Foucauldian Analysis - Beyond the Surface

In the Archaeology of the Knowledge, Foucault stated *that These problems can be summarized in one word: the review of the document's value. There is no ambiguity: it is patently obvious that since the discipline of history exists, documents have been used, questioned, and questioned about them; not only what they wanted has been asked of them, but whether they told the truth well, and with what entitlement they could claim it; whether they were sincere or falsifiers, well informed or ignorant, authentic or altered. But each of these questions and all this great critical concern pointed to the same end: to reconstruct, from what these documents say - at times in half-words - the past from which they emanate and which has now faded far behind them; the document continued to be treated as the language of a voice now reduced to silence: its fragile trace, but fortunately decipherable.*[31] Foucault's insight distills historical analysis and interrogates documents to reconstruct the past. Nevertheless, the documents' truth is uncertain. Questioning their claims unveils their subjectivity. Nevertheless, amidst partiality, traces of the past emerge.

Foucault's insight on scrutinizing documents applies aptly to the citicoline debate. Like historians, we must critically analyze these scientific documents, aware that their truth is uncertain. Questioning the literature's claims unveils its subjectivity. Randomized trials, reviews, guidelines - all contain biases. Nevertheless, amidst their partiality, traces of truth regarding citicoline's efficacy emerge. We must dig beneath the surface to challenge the literature's assumptions to reconstruct an objective understanding. Foucault reminds us that documents reveal as much by what they conceal as what they share. Their silences speak volumes.

With a sharply critical eye, we can divine fragments of reality from even the most biased accounts. However, only by remembering that to find truth in documents, we must first question their truth[32]. Popper's principle of falsification also offers a critical perspective here. It encourages rigorously testing citicoline's efficacy claims against empirical evidence to see if they hold up to scrutiny. The combination of Foucault's document analysis and Popper's falsification provides a sharp lens to assess the citicoline evidence base.

Medawar's thoughts remain. Specifically, Medawar's emphasis on focusing scientific inquiry on "soluble" questions, which can advance understanding rather than getting stuck on insoluble problems, is tremendously valuable advice. As Medawar elegantly states: "There are questions which science cannot answer, and it is no good beating one's head against these questions. What matters is to learn to discriminate between soluble questions with real and accessible answers and questions that are beyond solution for the time being." It highlights the wisdom of channeling curiosity toward questions matched to current capabilities while appreciating present limitations. Progress lies in the pursuit of the soluble. Medawar also astutely notes that even incorrect answers can unveil fragments of truth and "expose the substratum on which truth rests." Science expands its edifice brick by brick through this spirit of imaginative yet grounded inquiry. These philosophical principles remain highly pertinent, broadly to research methodology and specifically to exploring controversial areas like the citicoline debate. Focusing on judiciously framed, tractable questions allows the gradual accretion of knowledge.

The systematic review process requires meticulously examining the literature, probing each study's methodology, assessing the risk of bias, and synthesizing the totality of evidence. It aligns closely with Foucault's emphasis on interrogating documents to reconstruct reality from their selective perspectives and inherent subjectivity. Unlike individual studies, which offer a narrow window, systematic reviews provide a panoramic view - meticulously surveying the landscape to discern the contours of truth. The protocols guard against bias by casting a wide net and pre-specifying rigorous inclusion criteria.

This systematic digging beneath the literature's surface allows faint signals to emerge from the noise. In this way, a scrupulous systematic review operationalizes Foucault's approach to document analysis. It crystallizes his ideals of questioning claims, unpacking assumptions, and piecing together truth from imperfect accounts. When rigorously conducted, the review methodology filters out bias to reveal the tentative insights documents can offer when probed critically. In short, the systematic review, emphasizing comprehensive searching, objective appraisal, and synthesis, epitomizes the spirit of interrogating truth in documents that Foucault advocates. It constructs meaning from complexity. Of note, the randomized controlled trial with the largest sample size, which was industry-sponsored, found that citicoline was ineffective for treating moderate to severe acute ischemic stroke.[33]

8.1.4 Concluding Thoughts

In the journey of this essay, we have navigated a multidimensional landscape of scientific pluralism to address a fundamental question: Should citicoline not be prescribed for individuals with acute ischemic stroke?

Drawing from the robust framework of Evidence-Based Medicine, we have delved into the most rigorous research available, particularly the findings of the Cochrane systematic review focused on citicoline for acute ischemic stroke.

This pinnacle of evidence has provided a clear message: the efficacy of citicoline in this context remains uncertain at best.

This pinnacle of evidence has provided a clear message: the efficacy of citicoline in this context remains uncertain at best.

However, our analysis ventured beyond just the empirical data into the philosophical principles of scientific inquiry. Insights from Karl Popper reinforced the importance of the falsifiability of claims. When assessed through this lens, the case of citicoline faces obstacles.

As Peter Medawar wisely cautioned, research must focus on questions matched to current capabilities. Progress lies in the "soluble" while acknowledging present limitations. Although portions of citicoline's efficacy may one day be uncovered, current evidence raises barriers.

Bridging philosophy and evidence provides a multidimensional perspective on the risks of unproven treatments. The lure of new interventions can obscure the need for robust proof. As Michel Foucault illuminated, exertion of power underlies medical "truths."

In conclusion, the amalgamated scientific and philosophical insights provide compelling grounds to question the justification of citicoline for acute ischemic stroke. While hope persists, judicious skepticism prevails given the current evidence favoring alternatives firmly proven effective.

Colophon

In closing, I am compelled to ask: Where is the definitive scientific evidence to support prescribing citicoline for acute ischemic stroke? The corpus of research contains abundant, consistent proofs that do not support using citicoline as a medical food for this indication. The lack of compelling proof of benefits, paired with philosophical principles of truth-seeking, gives me pause. Only when convincing positive evidence emerges will the science point away from prescribing citicoline for acute ischemic stroke, directing me towards proven options. While hope persists, I must align with the evidence. In the spirit of ethical, accountable inquiry, I remain open yet adequately skeptical.

Coda

Truth is ever to be found in simplicity, and not in the multiplicity and confusion of things. Isaac Newton.

The important thing is not to stop questioning. Curiosity has its own reason for existence. Albert Einstein.

Conflict of Interest

I declare that there are no conflicts of interest associated with the composition of this essay.

Statement: I used Chat GPT 4 Plus to create the figures 2 and 5.

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ABSTRACT

The present study analyzed the chemical constituents of the Methanol leaf extract of Hymenocardia acida (MLEHA), and its In-silico parameters. The MLEHA was obtained by Soxhlet extraction, and then subjected to Atomic Absorption Spectroscopy (AAS), High performance liquid chromatography (HPLC) and Gas Chromatography-Flame ionization detection (GC-FID). The ADME-T and molecular docking studies were performed on orientin and chromone from HPLC and GC-FID data, using rofecoxib and diclofenac as reference drugs. The MLEHA contains high levels of nickel, zinc, cobalt and iron. The HPLC depicts presence of 3- hydroxybenzoic acid, betulinic acid, orientin, beta- sitosterol, coumarin, stigmasterol, rutin, friedelin, chromon, squalene, lupeol and vitexin.

Keywords: elemental analysis, compound profiling, cyclooxygenases, Hymenocardia acida, in-silico studies.

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ABSTRACT

The present study analyzed the chemical constituents of the Methanol leaf extract of Hymenocardia acida (MLEHA), and its In-silico parameters. The MLEHA was obtained by Soxhlet extraction, and then subjected to Atomic Absorption Spectroscopy (AAS), High performance liquid chromatography (HPLC) and Gas Chromatography-Flame ionization detection (GC-FID). The ADME-T and molecular docking studies were performed on orientin and chromone from HPLC and GC-FID data, using rofecoxib and diclofenac as reference drugs. The MLEHA contains high levels of nickel, zinc, cobalt and iron. The HPLC depicts presence of 3-hydroxybenzoic acid, betulinic acid, orientin, beta- sitosterol, coumarin, stigmasterol, rutin, friedelin, chromon, squalene, lupeol and vitexin. whereas CG-FID shows presence of 3- hydroxy-benzoic acid, betulinic acid, oleic acid, orientin, coumarin, chromon, paviin, hymenocardine, homopterocarpin, stigmasterol, rutin, friedelin, squalene, vitexin, alpha- colubrin, chelidonin and anthatrone. The ADME-T analysis shows that orientin has the least Caco-2 permeability and highest p- glycoprotein inhibition among the four compounds. Orientin and chromone showed higher induction and lower inhibition potentials on Cytochrome-p450 enzymes relative to rofecoxib and diclofenac. Orientin showed lower carcinogenicity and mutagenicity than rofecoxib and diclofenac. Orientin, chromone, rofecoxib and diclofenac have binding energies of -2.3, -5.6, -5.7 and - 5.6 kcal/mol with COX-1, and -6.10, -7.0, -9.7 and -7.6 kcal/mol with COX-2, respectively. Orientin formed H-bonds with Asn80, His43 and Arg83, while diclofenac formed H-bonds with Gln461, Tyr130 and Cys41 of COX-1 enzyme. Orientin formed H-bonds with Glu524, Ser119(2), Arg120, whereas rofecoxib forms H-bonds with Arg513 and His90 of COX-2 enzyme. This study has shown that Hymenocardia acida leaf is rich in minerals and phytochemicals. Orientin could potentially inhibit Cyclooxygenases- 1 and 2 activities, which are involved in development of pains and inflammation.

Keywords: elemental analysis, compound profiling, cyclooxygenases, *Hymenocardia acida*, in-silico studies.

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I. INTRODUCTION

Phytochemicals have been used across the world for treatment of various diseases and ailments, and these agents serve as good sources of developing modern drugs.[1, 2] Plants that possess therapeutic or pharmacological potentials are designated as medicinal plants.[3] Medicinal plants are plants containing one or more active compounds with therapeutic potentials or

compounds that could be used for synthesis of useful drugs.[4]

In the African continent, *Hymenocardia acida* tul (Hymenocardiaceae) is a popular trado-medicinal plant, with its leaves and stem bark being used in treatment of several diseases.[5, 6] The plant grows as a shrub in both savannah and deciduous woodland areas.. The plant can thrive well on clayey, laomy and sandy soils, covering up to a height of 9 m.[7] As documented by Bum *et al*,[8] the genus, hymenocardia belongs to a distinct faminly in the genera of Euphorbiaceae. Tor-Anyiin *et al* [1] and Ibrahim *et al*[9] documented that in Nigeria, the plant is called by different names based on tribes. For instance, Enanche (Idoma),, Ikalaga (Igbo), Ii-kwarto (Tiv), Uchuo (Igede), Orupa (Yoruba), Yawasatoje (Fulfude) and emela (Etulo).

Udeozo *et al*[10] noted that *H. acida* contains chemical constituents, including lignin, cellulose and hemicelluloses. Phytochemicals in methanol extract of *H. acida* include steroids, phenols flavonoids and, triterpenoids.[11] Methanol leaf extract of *H. acida* was reported to inhibit tracheal smooth muscle contraction, while the analgesic potential of the root bark was noted by Olotu *et al*.[12] A series of studies carried out by Ibrahim *et al*.[9] Tor-Anyiin *et* al[1] and Starks *et al* [13] revealed the ethno-medicinal applications of *H. acida* against hemorroids, eye infection, skin diseases, chest pains, migraine and tumors.

Adedokun *et al*[14] documented that *Hymenocardia acida* exerts anticancer potential, while Skovronsky *et al*[15] reported the activity of the plant against neurodegenerative diseases. *H. Acida* has been reported to possess activities against *Streptococcus pyrogens*, *Staphylococcus auricularis*, *S. aureus*, *Bacillus subtilis* and *Streptococcus mutans,* as well as *Candida albinicans* and *Aspergilus flavus*.[16] *H. acida* has shown potential to ameliorate. Sofidiya *et al*[11] reported the antioxidant activity of leaf extract of *H. acida* leaves, could possibly be due to presence of various phytochemicals.

An investigation carried out by Koffi *et al* [17] using rodents, noted that an intravenous injection aqueous extract of *H. acida* roots at a sub-chronic level, was non-toxic up to 1000 mg/kg, but could be harmful at higher doses. Acute toxicological studies of methanol extracts of leaf and root bark of *H. acida* revealed no mortality at doses up to 2000 mg/kg.[18] Both acute and sub-chronic effects of ethanol extract of *H. acida* leaf were investigated in rats by Obidike *et al*.[19] They reported that although, there was no hematological toxicity, the serum level of triglyceride was increased, with mild cortical-tubular cellular edema in kidneys of the experimental animals at the sub-chronic level. In this present study, we investigated the elements and various phytochemicals in methanol leaf extract of *Hymenocardia acida*, as well as the toxicological profile in spleen of Wistar rats exposed to different doses of the extract.

Cyclooxygenases (COX-1 and COX- 2) are membrane-bound enzymes implicated in the biosynthesis of prostanoids, including prostacyclins, prostaglandins and thromboxanes, which are all involved in important physiologic and pathologic processes.[20, 21] COX-1 (constitutive) and COX-2 (inducible) isozymes have been documented to have up to 67% similarity in amino acid composition, and a major disparity is in the presence of isoleucine (Ile523) in former instead of valine (Val523) in latter.[22] Although COX-1 is present in most tissues, the enzyme has been reported to play major roles in maintaining the physiologic functions of cardiovascular and gastrointestinal tissues.[23] When drugs which mainly inhibit COX-1 enzyme are used for a long period of time, there are usually adverse effects in the GIT, renal and hepatic tissues.[24] However, COX-2 is usually found over expressed during inflammation and many other pathological conditions.[25] Animal model studies have indicated that potential inhibitors of COX-2, are also capable of preventing cancer progression, hence can be used as potential anti-cancer agents.[26-28]

The present study was targeted to chemically characterize the methanol extract of *Hymenocardia acida* leaf, and carry out some computational studies on the effects of two constituents, orientin and chromone, of the

extract on the activities of cyclooxygenases – 1 and 2 isoforms.

II. MATERIALS AND METHODS

2.1 Collection and Extraction of Hymenocardia Acida Leaves

Leaves of *Hymenocardia acida* were collected in February, 2021, from the Ladoke Akintola University of Technology Campus, Ogbomoso, Oyo State, Nigeria. The leaves were air-dried for about 3 weeks, and pulverized with a mechanical grinder. Soxhlet extraction with methanol was carried out, followed by rotary evaporation and oven-drying, to obtain the Methanol leaf extract of *Hymenocardia acida* (MLEHA).

2.2 Elemental Analysis by Atomic Absorption Spectroscopy

The concentrations of Cobalt, Copper, Zinc, Iron, Nickel, Manganese, Magnesium and Chromium of MLEHA were determined by Atomic Absorption Spectroscopy.

2.3 Fourier-Transform Infrared Spectroscopy

The MLEHA was analyzed using FT-IR (Agilent Cary 630 FTIR spectrophotometer). Wavelength was expressed in reciprocal centimeter (cm^{-1}). Spectral values obtained were compared with literature data.

2.4 High-Performance Liquid Chromatography

The phytochemical profiling of MLEHA was determined with an isocratic HPLC machine (Mumbai, India) at a flow rate of 0.5 mL/min. Up to 25 mg of MLEHA was dissolved in a mixture of acetonitrile and methanol (80:20, v/v) as the mobile phase, at an injection volume of 20µL. The C18 (4.5 x 250 mm, 5µm) column was maintained at the room temperature and the eluent was detected at 210nm with a run time of 30 minutes. The inbuilt standard available in the NIST 11 library was used to compare the peaks obtained.

2.5 Gas Chromatography-Flame ionization detection Analysis

The phytochemicals of MLEHA were analyzed using a GC-FID machine (HP SERIES II- 5890) coupled to a flame ionization detector. The carrier gas was nitrogen maintained at a flow rate of 20 ml/min, while the combustion gas was hydrogen/compressed air at the flow rate of 45 ml/min. The initial, injector and detector temperatures were 50°C, 220°C and 270°C, respectively, while the oven was maintained at 240°C at the rate of 10°C/min, with a holding time of 2 minutes**.** Identification of the constituents was achieved by comparing the mass spectra with the standard available in the NIST 11 library. The peak area of each constituent was used to estimate the percentage composition.

2.6 ADME-T Analysis and Lipinski Test

ADME-T analysis was done using idrug webserver, to predict the drug-ability of two constituents of MLEHA (Orientin and Chromon) as shown from the HPLC and GC-FID results. Rofecoxib and Diclofenac were used as reference ligands. The four compounds were subjected to Lipinski test.[29]

2.7 Ligand Generation and Preparation

The Canonical SMILES of Orientin, Chromon, Rofecoxib and Diclofenac were gotten from a public database. Their SMILES (Simplified Molecular-Input Line-Entry System) format was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). They were imported into the LigPrep panel of Maestro version 13.4 (Schrödinger Release 2022-4: LigPrep, Schrödinger, LLC, New York, NY, 2022) for preparation. The compounds were optimized with an OPLS4 force field and tautomers were generated for each ligand using Epik at a target pH of 7.0 ± 2.0. Specified chirality was retained for stereoisomers, while the other stereogenic centers were varied to get a maximum of 5 isomers per ligand. Finally, an additional total number of the compounds was prepared (this happened because tautomers were generated for some molecules) and were used for docking analysis.

2.8 Protein Preparation and Grid Generation

The crystal structures of human cyclooxygenase- 1 (COX-1) and cyclooxygenase-2 (COX-2) were obtained from protein data hub - RCSB PDB (https://www.rcsb.org/) with an high atomic resolution of 270 Å. The protein structures were prepared and bond orders were assigned using the protein preparation wizard of Maestro version 13.4 (Schrödinger Release 2022-4: Maestro, Schrödinger, LLC, New York, NY, 2020).

Hydrogen atoms were added, filling the missing side chains using prime. The COX-1 and COX -2 structures were optimized and minimized (using the OPLS3e force field), and histidine residue protonation statuses were assigned using PROPKA at a pH of 7.0. A receptor grid was done to specify the active site of the proteins by selecting the active site generated from the Computed Atlas for Surface Topology of Proteins (CASTp) web server, and the grid box coordination for the proteins was generated.

2.9 Molecular Docking Studies

Molecular docking was done to evaluate the binding nature of Orientin, Chromon, Rofecoxib and Diclofenac within the biding pocket (active region) of receptors (COX-1 and COX- 2), using the virtual screening Schrodinger.

III. RESULTS

In figure 1, the AAS analysis of Methanol leaf extract of *Hymenocardia acida* shows the presence of cobalt (1.120 ppm), copper (0.370 ppm), zinc (1.219 ppm), iron (1.115 ppm), nickel (1.247 ppm), manganese (0.258 ppm), magnesium (0.412 ppm) and chromium (0.363 ppm). Subjecting the MLEHA to FT-IR spectroscopy, five peaks were revealed to be 1375.4, 1459.3, 2851.4, 2920.4 and 2958.5 cm^{-1}, which indicate C-H bond, aromatic ring, aldehyde group, carbonyl group and amine N-H stretching, respectively (Figure 2).

On compound identification of MLEHA by HPLC (Figure 3), twelve chemical constituents (here indicated with their retention times and percentage areas), were revealed as 3-hydroxybenzoic acid (1.350 min, 1.47%), betulinic acid (1.650 min, 2.47%), orientin (1.983 min, 64.34%), beta- sitosterol (3.166 min, 8.94%), coumarin (4.016 min, 8.24%), stigmasterol (6.350 min, 2.28%), rutin (7.350 min, 9.28%), friedelin (8.183min, 0.93%), chromone (8.616 min, 0.44%), squalene (9.250 min,0.40%), lupeol (9.750 min, 0.36%) and vitexin (10.266 min, 0.86%). However, the Gas Chromatography – Flame Ionization Detection spectrum (Figure 4) shows the presence of 3- hydroxybenzoic acid (3.300 min, 3.22%), betulinic acid (4.033 min, 1.57%), oleic acid (4.316 min, 0.81%), orientin (5.016 min, 17.29%), coumarin (6.033 min, 0.40%), chromone (6.450 min, 0.89%0, paviin (7.150 min, 2.41%), hymenocardine (7.533 min, 8.71%), homopterocarpin (7.816 min, 9.28%), stigmasterol (8.600 min, 10.59%), rutin (9.200 min, 39.94%), friedelin (11.061 min, 1.00%), squalene (11.483 min, 2.64%), vitexin (11.91 min, 0.32%), alpha- colubrin (12.233 min, 0.11%), chelidonin (12.550 min, 0.43%) and anthatrone (14.566 min, 0.36%).

Table 1 shows the results on ADME-T analysis, which tested the drug-likeness of the four ligands. Orientin has the least Caco-2 permeability (0.63%) and highest p- glycoprotein inhibition (0.92) values among the four ligands. Orientin (13%) has lower oral bioavailability than chromone (65%), while rofecoxib and diclofenac were found to be 86% and 94%, respectively.

Permeability glycoprotein (P-gp) inhibition was found to be highest with orientin (0.92) as against chromone (0.1), rofecoxib (0.01) and diclofenac (0.01) (Table 1). Plasma protein binding values of orientin (85%) and chromone (76%) were high comparable to both rofecoxib (90%) and diclofenac (96%). Blood Brain Barrier (BBB) permeability value of orientin (0.08) was less than that of chromone (0.72). The metabolism of the ligands was also tested, and the result is presented in table 1. Orientin (0.92) and chromone (0.88) have higher potential to induce CYP-450 enzymes relative to the two reference drugs. Orientin and chromone show far lower inhibition of CYP2C19, CYP2C9, CYP2D6 and CYP3A4 relative to both rofecoxib and diclofenac, as depicted from the ADME-T test. Orientin (0.82) was found to show

higher body clearance relative to chromone (0.30), rofecoxib (0.24) and diclofenac (0.47) (Table 1). Toxicity testing of the four ligands shows that orientin (0.48) and chromone (0.44) have low hepatic toxicity relative to the reference drugs. Orientin was also found to show slightly lower carcinogenicity (0.18) and mutagenicity (0.02) than rofecoxib and diclofenac. However, chromone was found to show relatively high carcinogenicity (0.59) and mutagenicity (0.92) potentials (Table 1). The result of the Lipinski's rule of five (RO5) has been presented in table 2.

Orientin has far greater numbers of hydrogen bond acceptors (11) and hydrogen bond donors (8) than chromone, rofecoxib and diclofenac. However, the number of violation by orientin was 2, whereas the three other ligands have zero violation of RO5.

Molecular docking of the four ligands was performed to show the binding affinity, H-Bond and others possible interactions in the pockets of both COX-1 and COX-2 isoforms. The binding energies of orientin, chromone, rofecoxib and diclofenac with COX-1 were found to be -2.3, -5.6, -5.7 and - 5.6 kcal/mol, respectively (Table 3).

Orientin forms H-Bond interactions with Asn80, His43 and Arg83, while His43 residue of the enzyme forms two pi (Π) interactions with phenyl rings of the ligand, Arg83 forms a Π-Cation bond with a phenyl ring of orientin when docked with COX-1enzyme (Figure 5). The keto group of chromone forms a hydrogen bond with Cys47 (Figure 6), while Gln44 forms a hydrogen bond with a sulfoxy group in rofecoxib (Figure 7).

Diclofenac forms H-bonds with Gln461, Tyr130 and Cys41 in the binding pocket of COX-1 enzyme (Figure 8). Orientin, chromon, rofecoxib and diclofenac have the binding affinities of -6.10, -7.0, -9.7 and -7.6 kcal/mol, respectively, when docked inside the pocket of COX-2 enzyme (Table 3). Orientin forms H-bond interactions with Glu524, Ser119(2), Arg120, while two phenyl rings in this ligand form pi (Π) interactions with Tyr115(2) of COX -2 (Figure 9). Chromone (Figure 10) and diclofenac (Figure 12) form no interaction, while the sulfoxy group of rofecoxib forms H-bonds with Arg513 and His90 (Figure 11) in the binding pocket of COX-2 enzyme.

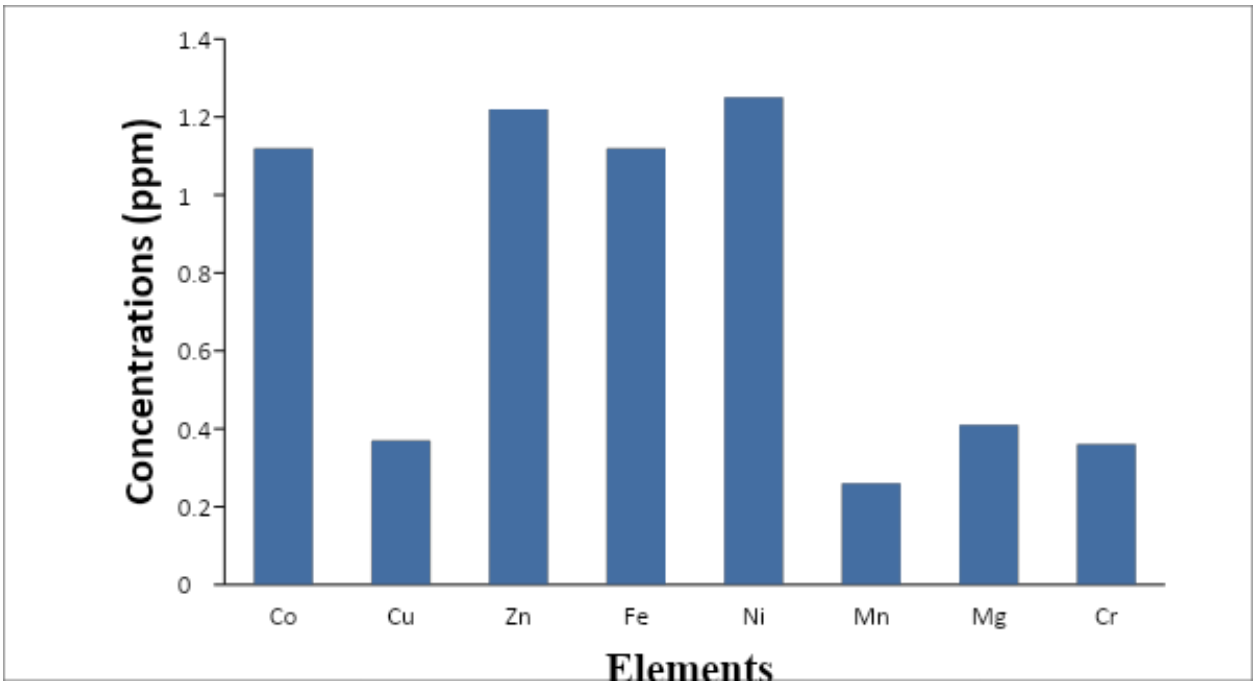

Figure 1: Elemental Composition of Methanol Leaf Extract of Hymenocardia Acida Using AAS

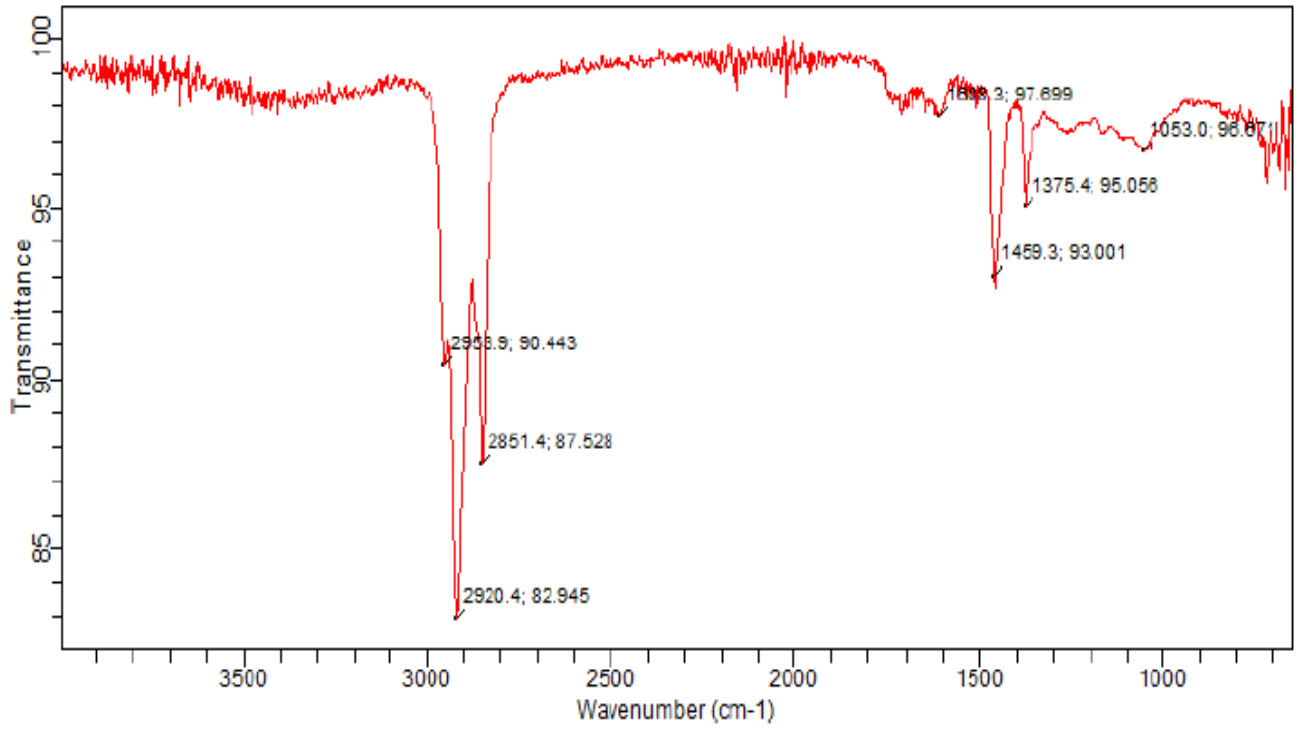

Figure 2: FT-IR Spectrum of Methanol Leaf Extract of Hymenocardia Acida

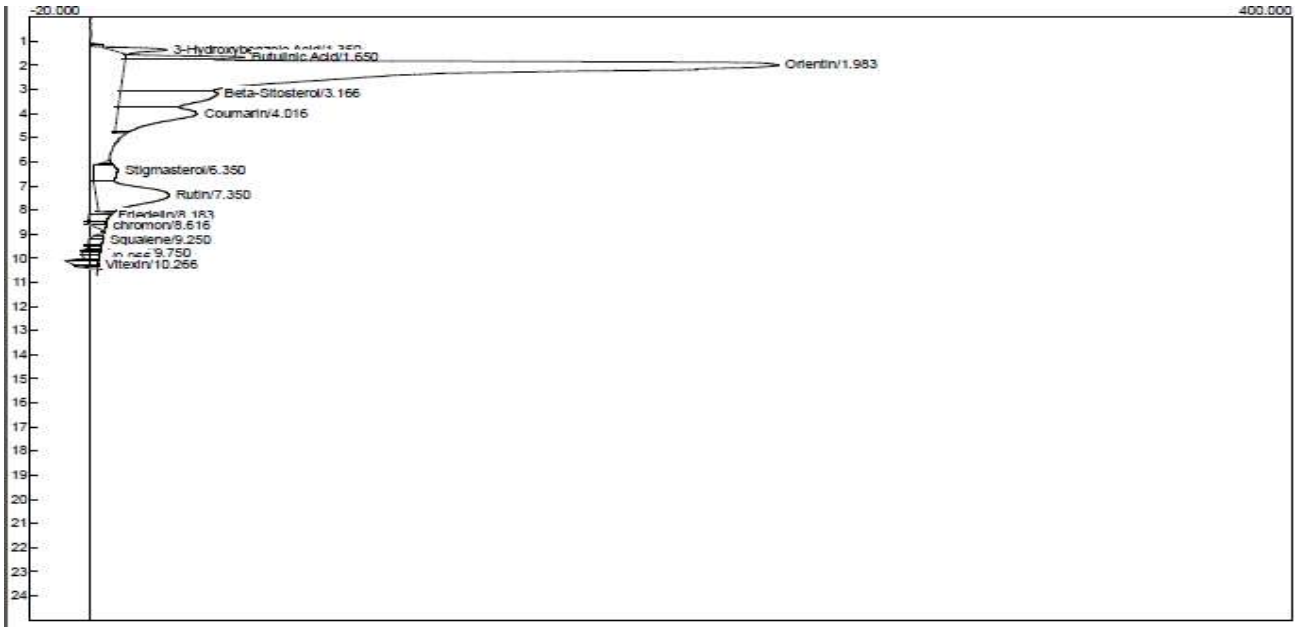

Figure 3: HPLC Chromatogram of Methanol Leaf Extract of Hymenocardia Acida

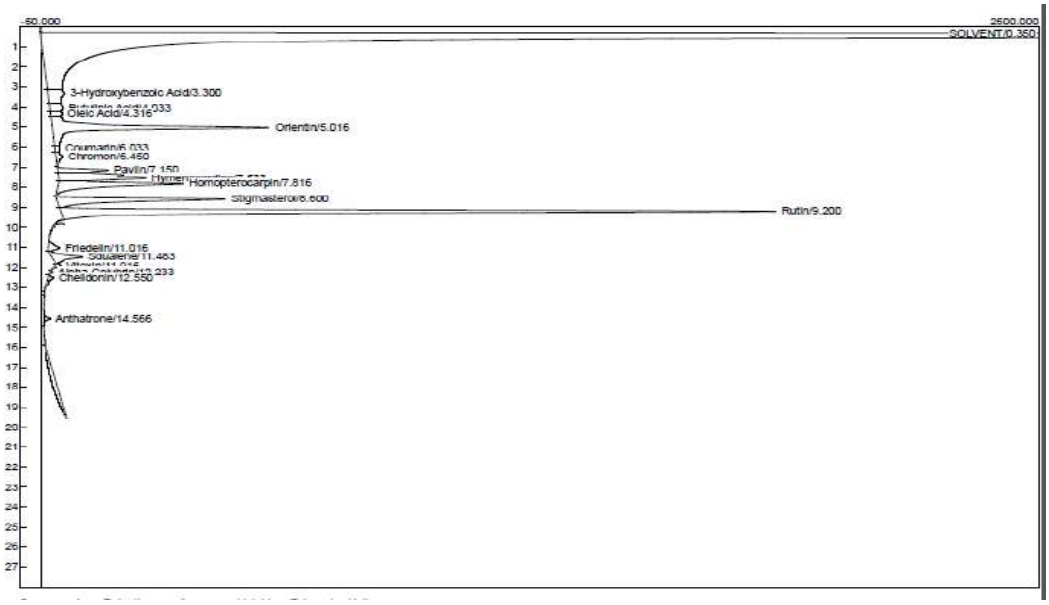

Figure 4: GC-FID Chromatogram of Methanol Leaf Extract of Hymenocardia Acida

Table 1: ADME-T Properties of Orientin, Chromon, Rofecoxib and Diclofenac

Properties		Orientin	Chromone	Rofecoxib	Diclofenac
Absorption	Caco-2 Permeability	0.63	36.54	29.33	38.20
	P-gp inhibition	0.92	0.10	0.01	0.01
	HIA	0.01	1.00	1.00	1.00
	Oral bioavailability (%)	13	65	86	94
Distribution	Plasma protein binding (Human) (%)	85	76	90	96
	Blood-Brain Barrier Permeability Probability	0.08	0.72	0.75	0.26
Metabolism	CYP Induction Probability	0.92	0.88	0.33	0.18
	CYP 1A2 Inhibition	0.66	0.99	0.93	0.62
	CYP2C19 Inhibition	0.30	0.25	0.89	0.53
	CYP2C9 Inhibition	0.28	0.24	0.92	0.64
	CYP2D6 Inhibition	0.06	0.26	0.82	0.36
	CYP3A4 Inhibition	0.00	0.03	0.36	0.55
Excretion	Human clearance	0.82	0.30	0.24	0.47
Toxicity	hERG Inhibition	0.01	0.01	0.00	0.00
	Ames Toxicity	0.81	0.20	0.04	0.04
	Hek293 Toxicity	0.10	0.11	0.05	0.04
	Hepatic Toxicity	0.48	0.44	0.90	0.95
	DILI	0.41	0.24	0.87	0.93
	Genotoxicity	0.93	0.61	0.88	0.79
	Carcinogenecity	0.18	0.59	0.33	0.30
	Mutagenicity	0.02	0.92	0.10	0.17
	Phospholipidosis	0.60	0.66	0.27	0.44

Table 2: Lipinski's Rule Profile of Orientin, Chromone, Rofecoxib and Diclofenac

Compound	Molecular weight (g/mol.)	H-Bond acceptor (< 10)	H-Bond donor (< 5)	miloP (< 5)	Number of violation
Orientin	448.38	11	8	-0.2	2
Chromon	146.14	2	0	1.79	0
Rofecoxib	314.36	4	0	2.56	0
Diclofenac	296.15	2	2	4.36	0

Table 3: Binding Profiles of Orientin, Chromone, Rofecoxib and Diclofenac With COX- 1 and COX- 2 Enzymes

COX- 1			
Compound	Binding energy (kcal/mol)	Hydrogen bond	Other bonds
Orientin	-2.3	Asn80, His43, Arg83	His43(2),Arg83
Chromone	-5.6	Cys47	None
Rofecoxib	-5.7	Gln44	None
Diclofenac	-5.6	Gln461,Tyr130,Cys41	None
COX- 2			
Compound	Binding affinity	Hydrogen bond	Other bonds
Orientin	-6.10	Glu524,Ser119(2),Arg120	Tyr115(2)
Chromone	-7.0	None	None
Rofecoxib	-9.7	Arg513,His90	None
Diclofenac	-7.6	None	None

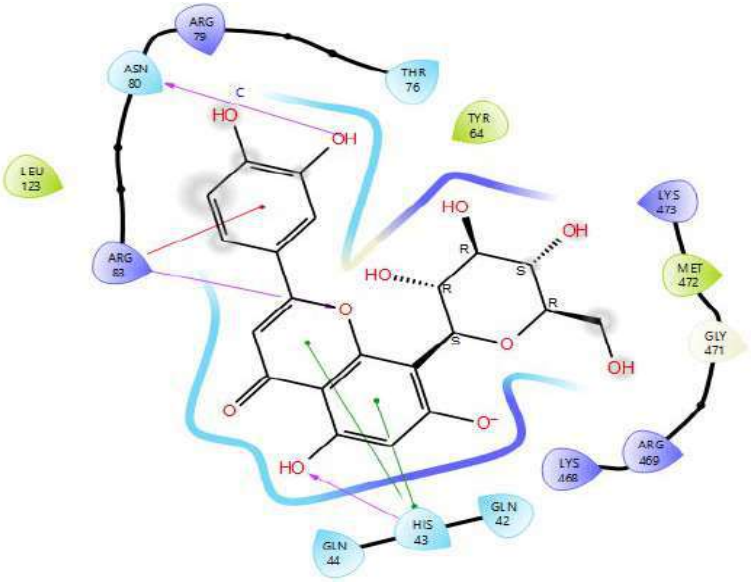

Figure 5: Molecular Docking of Orientin against COX-1 Enzyme

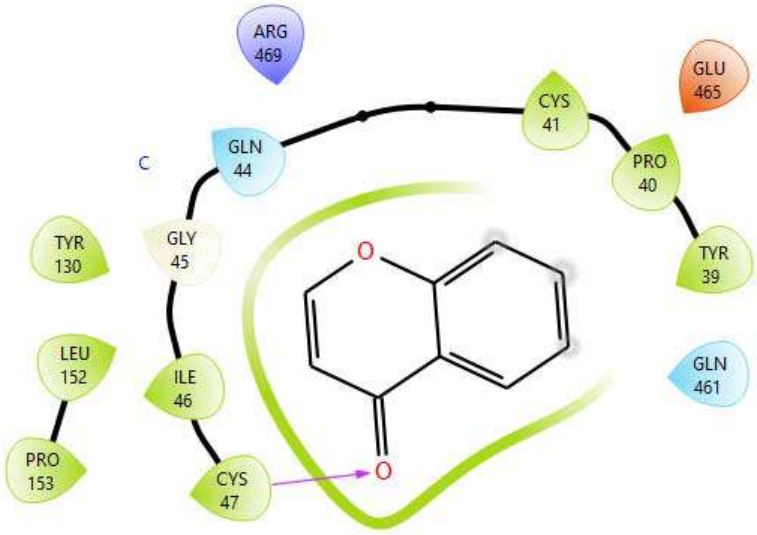

Figure 6: Molecular Docking of Chromone against COX-1 Enzyme

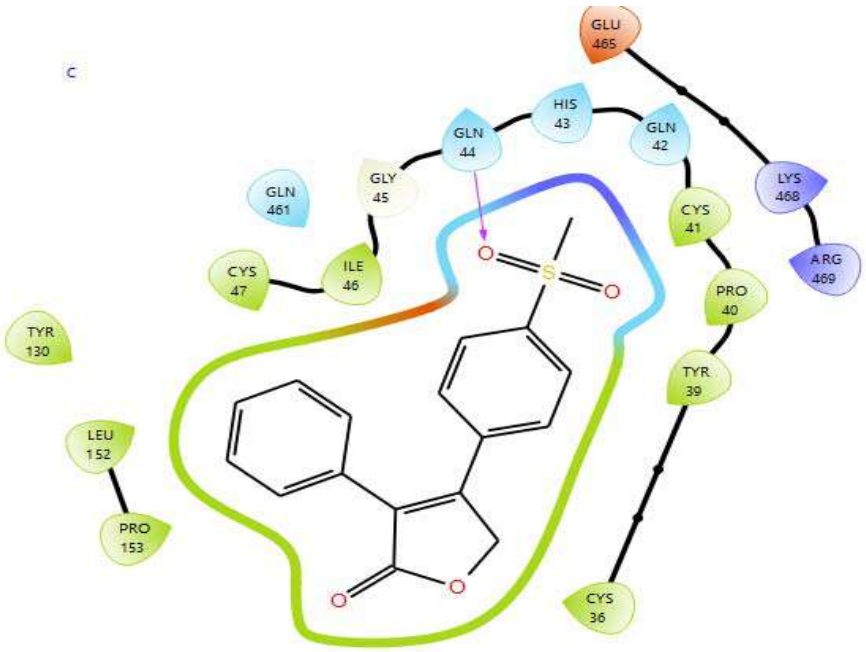

Figure 7: Molecular Docking of Rofecoxib Against COX-1 Enzyme

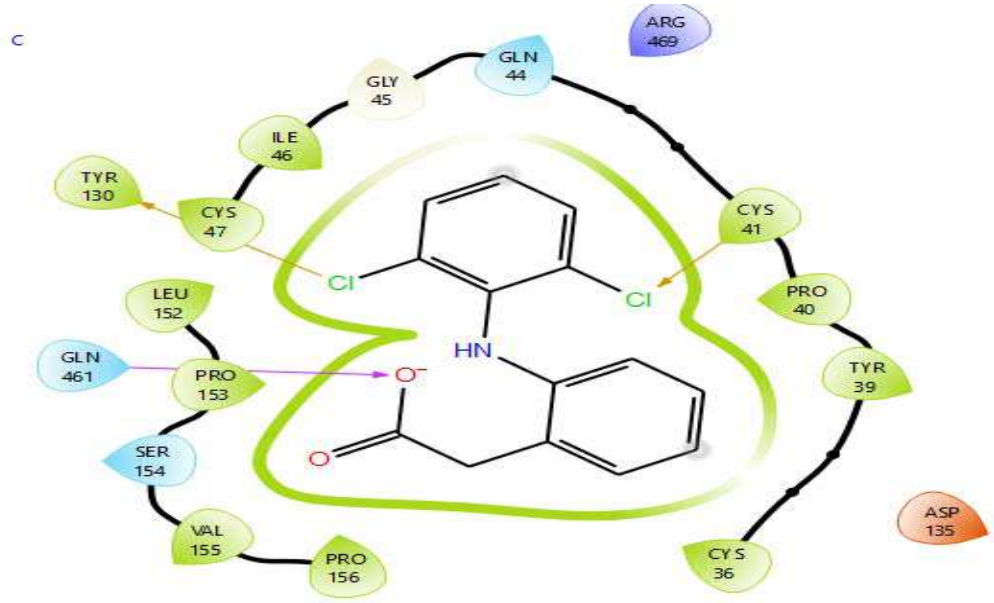

Figure 8: Molecular Docking of Diclofenac Against COX-1 Enzyme

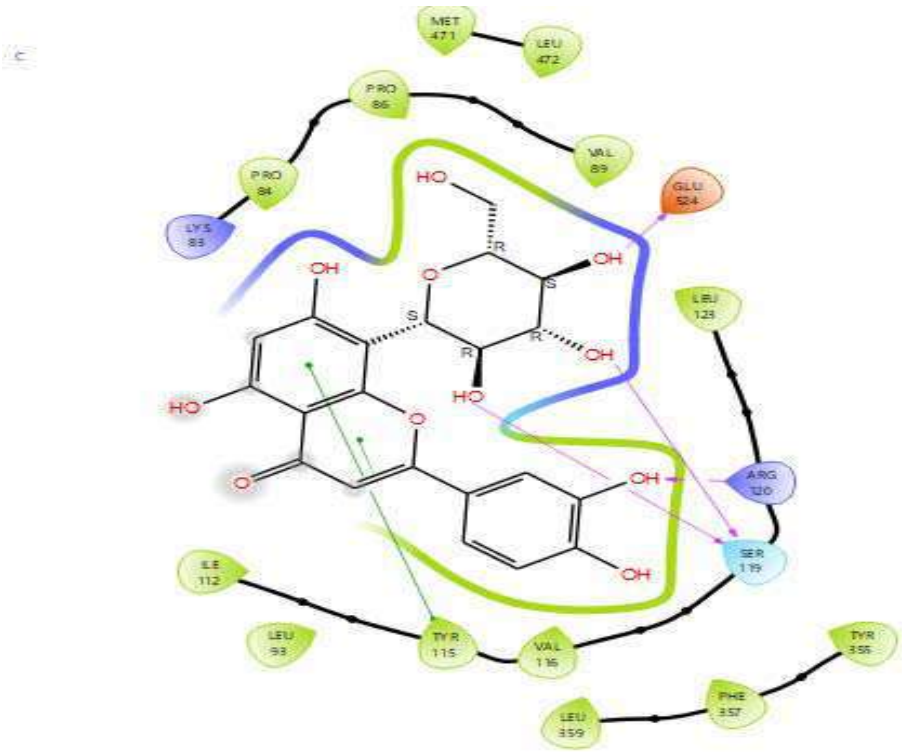

Figure 9: Molecular Docking of Orientin Against COX-2 Enzyme

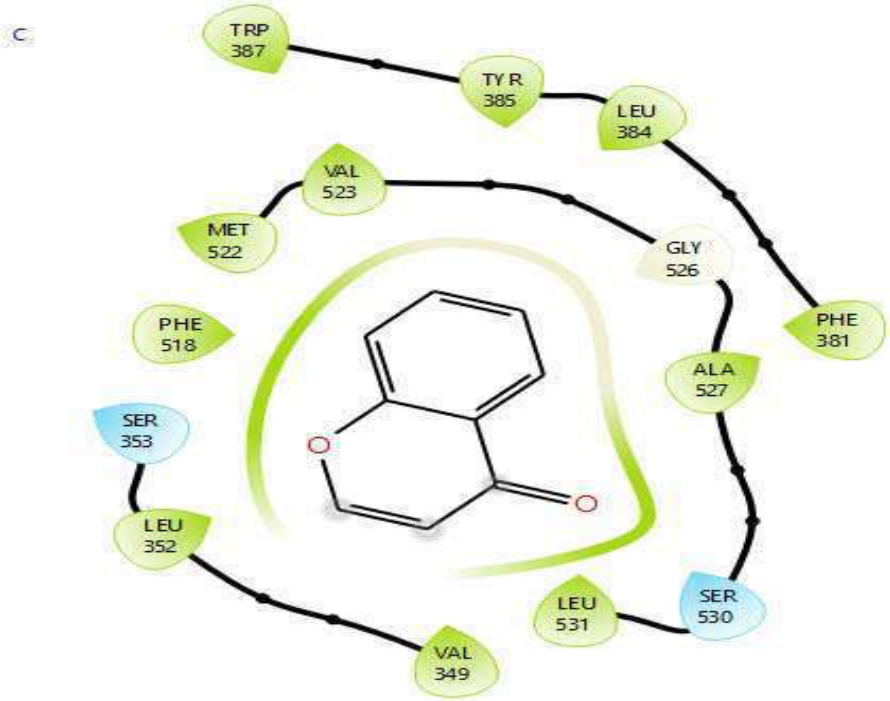

Figure 10: Molecular Docking of Chromon Against COX-2 Enzyme

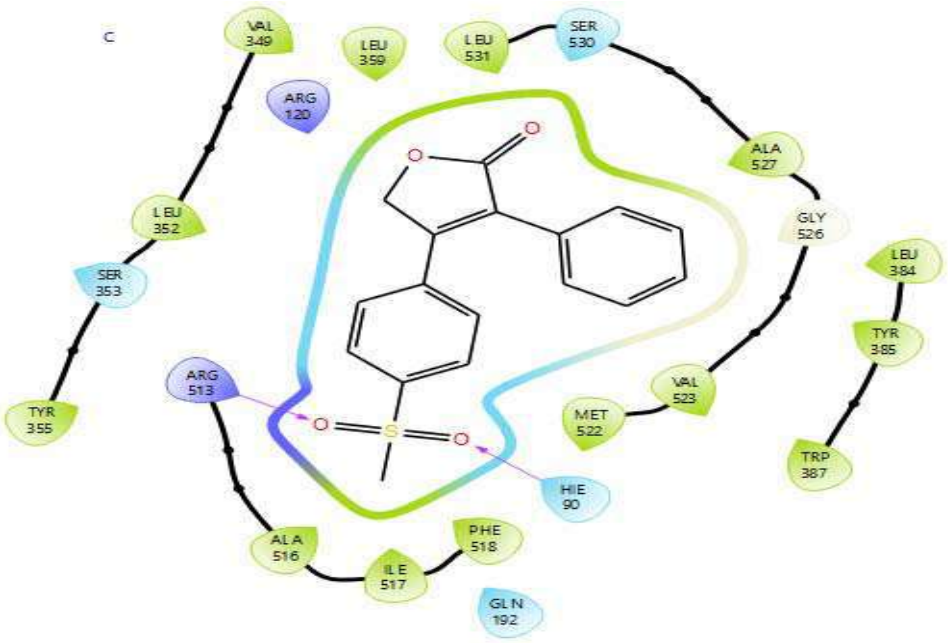

Figure 11: Molecular Docking of Rofexicob Against COX-2 Enzyme

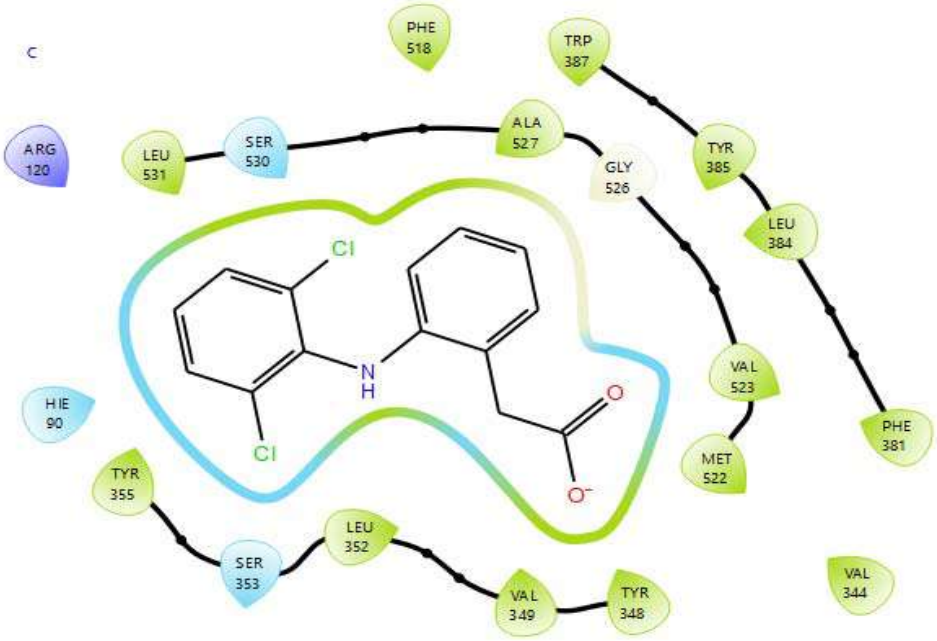

Figure 12: Molecular Docking of Diclofenac Against COX-2 Enzyme

IV. DISCUSSION

This study investigated the phytochemical nature of methanol extract of *Hymenocardia acida* leaf, as well as in-silico effects of orientin and chromone, two of the compounds identified in the extract, on activities of COX – 1 and 2 enzymes.

The AAS investigation has revealed that MLEHA contains high levels of nickel, zinc, cobalt and iron, while copper, manganese, magnesium and chromium are relatively low in concentration. In agreement with our present observation, Udeozo *et al*[10] detected magnesium, copper and zinc in a methanol extract of *H. acida*. Our recent study has indicated that the tree bark of *H. acida* contains the same elements detected in the present investigation.[30] Iron improves heart failure and exercise intolerance,[31-33] nickel improves dyslipidemia,[34] while zinc improves cellular homeostasis of zinc in humans.[35] The

presence of the various elements in the extract, as observed in this study, thus suggests a great physiologic significance of *Hymenocardia acida* leaf extract as a supplement. The FT-IR spectral data indicates the presence of C-H bond (1375.4 cm^{-1}), aromatic ring (1459.3 cm^{-1}), aldehyde group (2851.4 cm^{-1}), carbonyl group (2920.4 cm^{-1}) and amine N-H stretching (2958.5 cm^{-1}) as documented by Fessenden and Fessenden[36] and Shriner *et al.* [37] A study carried out by Adedokun *et al* [14] revealed that FT-IR spectral data of *H. acida* stem bark extract to be 1600 cm^{-1} and range between 3100 and 3600 cm^{-1}. Our findings in the present study is in agreement with that of Udeozo *et al*,[10] who reported the presence of functional groups including aromatic ring, carbonyl group and methyl stretching on compounds in an extract of *H. acida* stem bark.

The HPLC analysis revealed that MLEHA contains which are 3- hydroxybenzoic acid, betulinic acid, orientin, beta- sitosterol, coumarin, stigmasterol, rutin, friedelin, chromone, squalene, lupeol and vitexin. On Gas Chromatography – Flame Ionization Detection analysis, 3-hydroxybenzoic acid, betulinic acid, oleic acid, orientin, coumarin, chromone, paviin, hymeno-cardine, homopterocarpin, stigmasterol, rutin, friedelin, squalene, vitexin, alpha-colubrin, chelidonin and anthatrone. The various compounds quantified in MLEHA in this study have also been reported in our previous study to be present in the tree bark extract of the same plant.[30] In this investigation, orientin has been found to have the largest proportion among the compounds in the extract. Orientin, a water-soluble flavonoid C-glycoside of luteolin, has antibacterial, cardioprotective, antiinoceptive, antidepressant and anti-inflammatory potentials, [38] and could prevent 1, 2-dimethylhydrazine-induced colonic cancer in rats.[39] Certain chromones isolated from *Dictyoloma vandellianum* have been shown to possess anti-inflammatory activity.[40] One of our studies in the recent time revelaed that the tree bark of *H. acida* could prevent cardiac and renal damage in rat model by inducing activities of antioxidant and carboxylesterase enzymes.[30] According to Fu *et al*, [41] orientin induces the nitric- oxide- cGMP pathway to vasodilate the thoracic aortic rings, while it causes muscle relaxation by activating the voltage – dependent calcium channels in New Zealand rabbit. The compound has also shown cardioprotective activity by suppressing the mitochondrial cytochrome C- caspase -3 apoptotic pathway in mycardiac tissue with ischaemia reperfusion in rat model.[42]

On subjecting orientin, chromone, rofecoxib and diclofenac to ADME-T study, orientin was found to have the least Caco-2 permeability and highest p- glycoprotein inhibition among the four compounds. The oral bioavailability of orientin was lower than that of chromone. Caco -2 cells are a human colon epithelial cancer cell line used as a model of human intestinal absorption of drugs and related compounds. [43] The pemeability of human Caco-2 cell monolayer to various molecules has been cartegorized as low (0 -20%), moderate (20 – 80%) and high (80 -100% fraction absorbed). A clinical significance of high Caco-2 cell permeability is an increased tendency for human oral bioavailability of the test compound.[44] The present study has revealed that orientin has low Caco-2 permeability, suggesting its reduced transport across the intestinal mucosal and low oral bioavailability. While a study by Liu *et al*[45] indicated that orientin and vitexin isolated from *Trollius chinensis* were hardly transported, Jian et al[46] have reported an involvement of passive diffusion in the transport of both orientin and isoorientin via Caco -2 cells. Paek *et al* [47] and Yan *et al*[48] have shown that glycosylation of compounds generally reduces their Caco-2 monolayer permeability, except a strong active transport is involved. However, Ahmed *et al*.[49] have shown that Caco-2 permeability could be enhanced by glycosylation, and that permeability assay is a suitable method for determining bioavailability of compounds from medicinal plants.

A very recent study has shown that orientin could inhibit the proliferation of glioblastoma and colon carcinoma cells in humans.[50] Permeability glycoprotein (P-gp) is a membrane transporter with the capacity to efflux drug molecules out of cancer cells, leading to failure in cancer chemotherapy. The P-gp inhibition value of

orientin was the highest relative to those of the other compounds. Presence of P-gp in the GIT and other sites for epithelial absorption has led to reduced oral bioavailability.[51] The high P-gp inhibition value of orientin in this study suggests a low level of cellular efflux of this compound.

Orientin and chromone showed high values of plasma protein binding comparable to the two reference drugs, while the Blood Brain Barrier (BBB) permeability of orientin was less than that of chromone. We found that orientin and chromone have higher potential to induce CYP-450 enzymes than both rofecoxib and diclofenac. However, the inhibitory potentials of orientin and chromone on CYP2C19, CYP2C9, CYP2D6 and CYP3A4 were lower than those of rofecoxib and diclofenac. The ADME-T analysis also indicated that orientin has a faster rate of body clearance than the other compounds, whereas, orientin and chromone showed lower hepatic toxicity than the reference drugs. Orientin showed lower risks of carcinogenicity and mutagenicity than chromone, and this possibly suggests a more promising status of the former than the latter, in drug discovery and development.

The Lipinski's RO5 has shown that orientin has the greatest number of hydrogen acceptors and donors among the four compounds investigated in this study, although its number of violation is 2.

Furthermore, this study has shown orientin to possess the least values of binding affinity for COX-1 and 2 among the four ligands. However, the overall binding profile of orientin is the most robust among the four compounds. This is evidenced from the ability of orientin to form a total of six interactions each with COX-1 and COX-2, relative to the three other compounds, forming less. This finding supports the trado-medical use of *H. acida* in treatment of several ailments as documented by Olotu *et al*,[12] Starks *et al*[13] and Adedokun *et al*.[14] Orientin has been found to inhibit the active region of COX-1 by forming hydrogen bonds with amino acid residues Asn80, His43 and Arg83, and other kinds of bond with His43 and Arg83 residues. Diclofenac was found to interact with the active region of the enzyme by forming hydrogen bonds with Gln461, Tyr130 and Cys41 residues. This finding shows that orientin and diclofenac may possess strong inhibition against CoX-1, relative to chromone and rofecoxib.

Keifer *et al* [52] documented that COX-2 has three specific regions in its active site. The first is a hydrophobic pocket lined with Tyr385, Trp387, Phe518, Ala201, Tyr248 and Leu352 amino acid residues. The second region is a hydrophilic entracne containing Arg120, Glu524 and Tyr355, while the third region is a hydrophilic side pocket which is made up of His90, Arg513 and Val523.

Our docking study has demonstrated that diclofenac could inhibit COX- 1,while rofecoxib could inhibit COX- 2. Orientin has shown inhibition (binding and affinity) against both COX-1 and 2, but more importantly, against COX-2 than COX-1, indicating a greater tendency of the compound to inhibit COX -2 more than COX -1. In the present study, we found that orientin interacts with Arg120 and Arg524 residues, which are located at the entrance of the active site of COX-2. Orientin also forms two hydrogen bonds with Ser119 residue, and two pi bonds with Tyr115 residue at the active site of COX- 2 enzyme. While chromone and diclofenac shows no inhibition against COX-2, rofecoxib demonstrates binding with Arg513 and His90, which are located in the side pocket of the active site of the enzyme. [52] The present study suggests that orientin inhibits COX-2 by blocking the entrance of the active site, whereas rofecoxib inhibits the enzyme by interacting with the amino acid residues in the side pocket of the active site. The safety of rofecoxib as an anti-inflammatory drug has been questioned due to its cardiovascular side effects, which led to its withdrawal from market in 2004.[53] Since overexpression of COX-2 isoform has been implicated in inflammation, cancers and certain other pathological conditions,[25 - 28] orientin could therefore be explored in developing safer and more potent drugs for treating these conditions. Furthermore, orientin, being a natural product, could be developed into novel drugs as replacements for traditional non-steroidal anti-inflammatory drugs (tNSAIDs), including

ibuprofen, lonazolac and aspirin, which have shortcomings like bleeding, hepatotoxicity and gastric ulceration.[54]

V. CONCLUSION

Our findings from this study have indicated that the leaf extract of *Hymenocardia acida* contains many physiologically importnat minerals and phytochemicals. Furthermore, orientin, one of the phytochemicals prensent in the leaf extract, shows promising potential to inhibit both cyclooxygenases - 1 and 2. This study raises a hope that orientin from the leaves of *H. acida* could be used in developing novel therapeutic agents for treating diseases involving the activities of COX -1 and COX -2 enzymes.

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