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Highlights

- Black Fungus in the Corona Virus
- Oxidative Stress in Wistar Albino Rats
- Formulation Method of Nanoparticles
- Evaluation of the Anthelmintic Activity

Discovering Thoughts, Inventing Future

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By Amadou Dicko, Almamy Konate, Arnaud S. R. Tapsoba, L. D. Dahourou, Moumouni Sanou, Adama Kabore, S. Tembely, Linda L. Logan, Amadou Traore, Balé Bayala & Hamidou H. Tamboura

Abstract- Medicinal plants with anthelmintic properties are an alternative to the chemical fight against small ruminant's gastrointestinal nematodes. In order to broaden the spectrum of medicinal plants with anthelmintic properties, ovicidal activity and L3 larvae exsheathment of *C. sesamoïdes* Endl and *S. hermonthica* (Deli) Benth, aqueous extracts has been done on *H contortus* egg and L3 larvae. Three concentrations, 3.12 mg / ml, 6.25 mg / ml, 12.5 mg / ml of each plant extract were used to assess the inhibition of fresh egg hatching and larval paralysis as well as the 'inhibition of L3 larvae exsheathment. A negative control (PBS1x) and a positive control (levamisole, 2.5 mg / ml) were constituted for the fresh egg hatching inhibition assessment while only the negative control (PBS1x) was constituted to evaluate the inhibition of L3 Larvae exsheathment.1 ml of each concentration was contacted with 1 ml of the egg solution and then incubated for 48 hours at 27 ° C in petri dishes (60X15 Cm) for hatching test inhibition.1 ml of each extract concentration was contacted with 1 ml of the embryonated egg solution after 24 hours of incubation at 27 ° C for the larval paralysis test.

Keywords: ceratothéca sesamoides endl, striga hermonthica (Deli.) benth, H. contortus, egg hatching inhibition, L3 larval exsheathment inhibition.

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Evaluation De L'activité Anthelminthique Des Extraits Aqueux De *Striga Hermonthica* (Del.) Benth Et *Ceratotheca Sésamoïdes* Endl Sur Deux Stades Biologiques De *Haemonchus Contortus*

Amadou Dicko [α], Almamy Konate [σ], Arnaud S. R. Tapsoba [ρ], L. D. Dahourou [ω], Moumouni Sanou [¥], Adama Kabore [§], S. Tembely [χ], Linda L. Logan [ν], Amadou Traore [Θ], Balé Bayala [ζ] & Hamidou H. Tamboura [£]

Résumé- Les plantes médicinales à propriétés anthelminthiques constituent une alternative à la lutte chimique contre les nématodes gastro-intestinaux des petits ruminants. Dans l'objectif d'élargir le spectre des plantes médicinales à propriétés anthelminthiques, une évaluation de l'activité ovicide et d'inhibition du dégainement des larves L3 de *H contortus*, des extraits aqueux de *C. sesamoïdes* Endl et *S. hermonthica* (Deli.) Benth a été effectuée. Trois concentrations, 3,12 mg/ml, 6,25 mg/ml, 12,5 mg/ml de chaque extrait de plante ont été utilisées pour évaluer l'inhibition de l'éclosion des œufs frais et de paralysie larvaire ainsi que l'inhibition du dégainement des larves L3. Un Témoin négatif (PBS1x) et un témoin positif (lévamisole, 2,5 mg/ml) ont été constitués pour l'évaluation de l'inhibition de l'éclosion alors que seulement le témoin négatif (PBS1x) a été constitué pour évaluer l'inhibition du dégainement des larves L3. 1 ml de chaque concentration a été mise en contact avec 1 ml de la solution d'œufs puis incuber pendant 48 heures à 27° C dans des boîtes de pétri (60X15 Cm) pour le test de l'inhibition de l'éclosion. 1 ml de chaque concentration d'extrait a été mise en contact avec 1 ml de la solution d'œuf embryonné après 24 heures d'incubation à 27° C pour le test sur la paralysie larvaire. 1 ml de la solution larvaire a été mise en contact avec 4 ml de chaque concentration de la solution d'extrait pendant 3 heures pour l'inhibition du dégainement des larves L3. Les résultats ont montré une inhibition de l'éclosion de 53,89% à la concentration de 12,5 mg/ml avec *C. sesamoïdes* Endl approximativement au témoin positif (Lévamisole) 56,49% alors que celui du *S. hermonthica* n'a été que de 40,72%. Le taux de paralysie larvaire a été de 51,11% et 60,56% respectivement pour *S. hermonthica* (Deli.) Benth et C. sesamoïdes Endl. L'inhibition du dégainement pour l'extrait aqueux de *C. sesamoïdes* Endl est de 65,33% à 12, 5 mg/ml et 64% à la même concentration pour *S. hermonthica* (Deli.) à Benth après 60 minutes.

Mots clés: ceratothéca sesamoides endl, striga hermonthica (Deli.) benth, H. contortus, inhibition éclosion œuf, inhibition dégainement larves L3.

Abstract- Medicinal plants with anthelmintic properties are an alternative to the chemical fight against small ruminant's gastrointestinal nematodes. In order to broaden the spectrum of medicinal plants with anthelmintic properties, ovicidal activity and L3 larvae exsheathment of *C. sesamoïdes* Endl and *S. hermonthica* (Deli) Benth, aqueous extracts has been done on *H contortus* egg and L3 larvae. Three concentrations, 3.12 mg / ml, 6.25 mg / ml, 12.5 mg / ml of each plant extract were used to assess the inhibition of fresh egg hatching and larval paralysis as well as the 'inhibition of L3 larvae exsheathment. A negative control (PBS1x) and a positive control (levamisole, 2.5 mg / ml) were constituted for the fresh egg hatching inhibition assessment while only the negative control (PBS1x) was constituted to evaluate the inhibition of L3 Larvae exsheathment.1 ml of each concentration was contacted with 1 ml of the egg solution and then incubated for 48 hours at 27 ° C in petri dishes (60X15 Cm) for hatching test inhibition.1 ml of each extract concentration was contacted with 1 ml of the embryonated egg solution after 24 hours of incubation at 27 ° C for the larval paralysis test. 1 ml of the larval solution was contacted with 4 ml of each concentration of the extract solution for L3 larvae exsheathment inhibition test. The results showed a hatching inhibition of 53.89% at the concentration of 12.5 mg / ml with *C. sesamoids* Endl aproximatly to the positive control (Levamisole) 56.49% whereas hatching inhibition of *S. hermonthica* was only of 40.72%. The larval paralysis rate was 51.11% and 60.56% respectively for *S. hermonthica* (Deli.) Benth and *C. sesamoïdes* Endl. The inhibition of L3 larvae exsheathment for the aqueous extract of *C. sesamoides* Endl is 65.33% at 12.5 mg / ml and 64% at the same concentration for *S. hermonthica* (Deli.) Benth after 60 minutes.

Keywords: ceratothéca sesamoides endl, striga hermonthica (Deli.) benth, H. contortus, egg hatching inhibition, L3 larval exsheathment inhibition.

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I. Introduction

Haemonchus contortus est l'un des nématodes gastro-intestinaux qui cause la plus importante contrainte dans l'élevage des petits ruminants (Mangi et al, 2006). En effet, la faune parasitaire du tractus digestif des petits ruminants est dominée par *H. contortus* de par sa fréquence et son intensité (Koné et al, 2005; Dedehou et al, 2014; Koffi et al, 2018). Comparativement aux autres espèces de nématodes, *H. contortus* est le parasite des petits ruminants le plus pathogène capable de provoquer des maladies aiguës et des fortes mortalités dans toutes les catégories des petits ruminants (Akkari et al, 2013 ; MbogningTayo et al, 2014). Ce parasite hématophage peut causer une anémie conduisant à la réduction de la productivité chez les petits ruminants (Burke et al, 2007).

La gestion des nématodes gastro-intestinaux des petits ruminants repose fortement sur l'utilisation des anthelminthiques de synthèses (Chartier et al, 2014). L'utilisation répétée de ces anthelminthiques de synthèse a entrainé l'apparition des résistances irréversibles des parasites à la majorité des familles des molécules anthelminthiques (Apala et al, 2020). Aussi, en plus de la difficulté d'accès de ces molécules anthelminthiques de synthèse aux éleveurs ruraux, il faut ajouter l'écotoxicité de ces molécules pour l'environnement (Dedehou et al, 2014).

Face à cette situation différentes alternatives aux molécules anthelminthiques dans le traitement des nématodes gastro-intestinaux sont envisagées. Parmi ces solutions alternatives il y a l'utilisation des plantes fourragères à propriétés anthelminthiques.

L'objectif de cette présente étude est d'évaluer *in vitro* l'activité ovicide et inhibitrice du dégainement des larves L3 de *H. contortus* des extraits aqueux du *Strigahermonthica* (Del.) Benth et de *C. sésamoïdes* Endl.

II. Matériel et Méthode

a) Milieu d'étude

Les tests *in vitro*de l'évaluation de l'activité ovicide et inhibitrice du dégainement larvaire ont été réalisés au Laboratoire de Biologie et santé Animale (LaBioSA) du Centre de Recherches Environnementales, Agricoles et de Formation (CREAF) de Kamboinsin.

b) Récoltes des plantes

Cératothéca sésamoïdes Endl et *Striga hermontica (Delile) Benth* ont été récoltées puis séchées à l'ombre entre fin Septembre et mi-octobre dans une salle à la température ambiante à la Direction Régionale de la Recherche Environnementale et Agricoles (DRREA-Sahel). Les plantes ont été identifiées à l'Herbier National du Burkina Faso (HNBF) respectivement sous les numéros 8758 et 8759.

c) Préparation des extraits

Les plantes entières des deux espèces ont été récoltées tôt le matin et séchées dans une salle à la DRREA-Sahel à la température ambiante pendant 2 semaines et acheminées au CREAF de Kamboinsin par la suite. Les échantillons de chaque plante ont été par la suite broyés en poudre. Une macération aqueuse (100 g de poudre dans 900 ml d'eau distillée) a été effectuée pendant 24 heures sous agitation mécanique à la température ambiante. Les macérations ont été ensuite filtrées et concentrées au congélateur. Les filtrats concentrés ont été par la suite lyophilisées.

d) Tests biologiques

i. Préparations des solutions mères

Les solutions mères de chaque extrait ont été préparées en diluant 0,25 g d'extrait de chaque plante dans 10ml du PBS 1X pour obtenir 25mg/ml de concentration pour chaque extrait. La solution a été homogénéisée à l'aide d'un sonicateur pendant 3 minutes. A partir de la solution mère trois autres solutions de concentrations inferieures qui seront utilisées à savoir 12,5 mg/ml et 6,25mg/ml et 3,12 mg/ml de chaque extrait ont été réalisées. Le lévamisole (anthelminthique de référence) à 2,5 mg/ml et PBS 1X ont été utilisé respectivement comme témoins positif et négatif.

ii. Récoltes des œufs

Les œufs ont été obtenu selon la méthode décrite par Kaboré. (2009). Tout d'abord, Les vers adultes ont été récoltés sur des caillettes des ovins fraichement abattus à l'abattoir de Kamboinsin. Ensuite, les femelles ont été triées puis enfin broyées légèrement dans un mortier en porcelaine. Le broyat a été filtrées avec des tamis de mailles décroissantes de 55 μm et 25 μm. La solution d'œufs obtenue a été réajustée à 100 œufs par millilitre.

iii. Obtention et Récoltes des larves infestantes L3

Les larves L3 ont été obtenues en cultivant pendant 14 jours à 31° C les œufs dans des fèces d'ovins préalablement stérilisés à l'étuve à 130° C pendant 24 heures et mélangés avec de la sciure du bois. Après 14 jours la culture a été montée dans un gaze puis déposé sur le dispositif de Baermann pour récolter les larves L3. La solution larvaire obtenue a été mise dans un bécher et déposée au congélateur pendant 3 heures. Les larves ayant migrées au fond ont été récupérées et centrifugées trois fois à 2000 trs/mns pendant 10 mns. La solution larvaire a été ensuite réajustée à 2000 larves/ ml.

iv. Test d'inhibition de l'éclosion

Le test d'inhibition de l'éclosion a été réalisé selon la méthode modifié de Kaboré. (2009). 1 ml de la solution larvaire réajustée à 100 œufs/ ml a été mise en contact avec 1 ml de chaque concentration d'extraits dans une boîte de pétri (60X15 Cm) puis incubé

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pendant 48 heures à 27° C. Après 48 heures quelques gouttes de lugol ont été ajoutées dans chaque boîte de pétri pour stopper l'évolution des œufs.

Le test a été répété trois fois avec trois réplicas pour chaque concentration par répétition.

Le nombre de larve et d'œufs non-éclos ont été évalués en déposant 50 µl entre lame et lamelle et observant au microscope à l'objectif X10.

Le pourcentage d'inhibition de l'éclosion des œufs a été calculé selon la formule suivante (Kaboré, 2009):

$$\text{Pourcentage d'inhibition (\%)} = 100(1-X1/X2)$$

X1: Nombre d'œufs éclos dans les extraits testés

X2: Nombre d'œufs éclos dans le témoin négatif

v. *Test d'embryonnement*

Le test d'embryonnement a été réalisé selon la méthode modifiée de Waboponé et al. (2006) décrite par Kaboré. (2009). 1ml de la solution d'œuf à 100 œufs/ml a été incubé dans des boîtes de pétri (60X15 Cm) à 27° C. 24 heures après, lorsque les larves L1 ont été transparentes et ont commencé à se mouvoir dans les enveloppes des œufs, 1 ml de chacune des concentration test de chaque extrait a été ajouté dans chacune des boîtes de pétri constituant le lot test. 1 ml du PBS 1X et 1 ml de lévamisol à 2,5 mg/ml ont été ajouté dans les boîtes de pétri pour tenir respectivement de témoin négatif et témoin positif. Après 6 heures de contact des extraits et des œufs embryonnés quelques gouttes de lugol ont été ajoutées dans chaque boîte de pétri pour stopper l'évolution des œufs.

Le test a été réalisé en trois répétition avec 3 réplicas pour chaque répétition.

Le taux de paralysie larvaire (TPL) a été déterminé selon la formule de D'Angelo et al. (2014):

$$TPL = 100 - \left[\left(\frac{\text{Nombre de Larves L1}}{\text{Nombre d'œufs embryonnés}}\right)*100\right]$$

vi. *Test d'inhibition du dégainement larvaire*

Le test de dégainement des larves L3 a été effectué selon la méthode décrite par Par Zabré. (2018) combiné à celle de Okombé.(2011) et Marion.(2015).

En plus des trois concentrations (3,12mg/ml; 6,25mg/ml; 12,5mg/ml) des extraits de chaque plante, et un témoin négatif (PBS) réalisées précédemment, une solution dite de Milton (javel 2,6% dilué dans du Nacl 0,9% au 1/300) a été réalisée.

1 ml de la solution larvaire a été incubé avec 4 ml de la solution d'extrait pendant 3h dans des boîtes de pétri (60X15Cm) et agité chaque 30 minute. Après 3h, 200 µl ont été prélevés de chacune des boites de pétri et mis en contact avec 200 µl de la solution de milton dans un tube eppendorf 2 ml. Le test a été répété 3 fois.

La cinétique de dégainement a été suivie au microscope X20 aux temps, T0, T20, T40 et T60mns après contact avec la solution de Milton en mettant 50 µl entre et lamelle et en comptant les larves dégainées et engainées.

Le pourcentage des larves dégainées a été calculé selon la formule suivante:

$$\text{Pourcentage des larves dégainées (\%)} = \left(\frac{\text{Nombre de larves dégainées}}{\text{Nombre total de larves}}\right)*100$$

e) Analyse des données

Les données ont été saisies sur le logiciel Excel 2016 qui a servi aux calculs des moyennes et Ecart Type. Les moyennes du taux d'inhibition des éclosions ainsi que celles du taux de paralysies larvaires et du dégainement larvaire ont été comparées à l'aide d'une analyse de variance (ANOVA) à une voie suivi d'une comparaison multiple de moyenne au seuil de 5% avec le Packages Rcmdr version 2.7-1 du logiciel R version 4.1.0.

Une régression linéaire par la méthode probit a été effectuée avec le logiciel SPSS STATISTICA 26 afin de déterminer les concentrations inhibitrices 50% de l'éclosion des œufs et celle qui engendre la paralysie larvaire des œufs embryonnées de 50%.

Le logiciel PRISM GraphPad Software 5.00.288 nous a servi de tracer les graphiques doses-réponses de l'évolution du dégainement des larves L3 en fonction du temps.

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III. Résultats

Tableau 1: Effet des extraits aqueux de *Striga hermonthica* (Del.) Benth et *Cératothéca Sésamoides* Endl sur l'éclosions des œufs de *Haemonchus contortus*

Pourcentage d'inhibition de l'éclosion des Œufs										
	Doses(mg/ml) /Extrait aqueux du *S hermonthica*(Del.) Benth					Doses (mg/ml) /Extrait aqueux *C. sésamoïdes*Endl				
Répétitions	3,12	6,25	12,5	T(-)	T(+)	3,12	6,25	12,5	T(-)	T(+)
R1	23,08	46,16	38,46	0	53,84	1,86	22,22	50	0	48,16
R1	5,88	21,58	52,94	0	64,71	17,65	29,41	60,79	0	42,35
R 3	15,38	23,08	30,76	0	53,84	21,05	22,81	50,88	0	78,95
Moyenne ±σ	14,78 ±8,61 ab	30,27 ±13,78 bc	40,72 ±11,26 cd	0 a	57,46 ±11,26 d	13,52 ±10,24 a	24,81 ±3,99 a	53,89 ±5,99 b	0 a	56,49 ±19,67 b

R: Répétitions, T (-): Témoin négatif (PBS: Phosphate-Buffered Saline1X), T(+): témoin positif (Lévamisole à 2,5 mg/ml), σ: Ecart-Type, (a, b, c) différences entre les colonnes pour chaque extrait, les colonnes avec les lettres distincts ont les moyennes de pourcentage d'inhibition statistiquement différentes entre elles au seuil de 5%.

Tableau 2: Valeurs des Concentrations entrainants l'inhibition de 50% d'éclosion des œufs obtenues à partir de l'équation de la droite de régression

	R^2 d'ajustement	a	b	CI50
*C. sesamoïdes*Endl	0,97	2,15	1,99	11,83 mg/ml
S. hermonthica (Deli.) Benth	0,87	1,19	0,08	14,93 mg/ml

a: Ordonnée à l'origine b: pente de la droite régression, CI50: Concentration qui inhibe 50% de l'éclosion des œufs

Le tableau 1 montre l'effet des extraits aqueux des plantes testées sur l'inhibition de l'éclosion des œufs de *H. contortus*.

L'analyse de ce tableau nous indique que les extraits aqueux des plantes testées ont une activité inhibitrice de l'éclosion des œufs moyenne voire faible.En effet, on remarque une activité inhibitrice légèrement supérieure ou égale à 50% de l'éclosion des œufs seulement à la dose la plus élevée qui est de 12,5 mg/ml. Aussi seulement l'extrait aqueux de *C. sesamoïdes* Endl a obtenu un taux moyen d'inhibition de 53,89% alors que celui de l'extrait aqueux du *S. hermonthica* (Del.) Benth est de 40,72%. Ce tableau nous indique aussi une faible activité inhibitrice du lévamisole (témoin positif) qui est de 56,49% en moyenne pour le test avec l'extrait aqueux de *C. sesamoïdes* Endl et 57,46% en moyenne avec l'extrait aqueux du *S. hermonthica* (Deli.) Benth.

Le tableau 2 nous indique les valeurs de la droite de régression qui nous a permis d'obtenir les concentrations inhibitrices des extraits des deux plantes testées.

Tableau 3: Effet des extraits aqueux de *Striga hermonthica* (Del.) Benth et *Cératothéca sésamoïdes* Endl sur la paralysie des larves L1 embryonnées de *Haemonchus contortus*

Pourcentage de paralysie larvaire des Œufs embryonnés										
	Doses(mg/ml) /Extrait aqueux du *S hermonthica*(Del.) Benth					Doses (mg/ml) /Extrait aqueux *C. sésamoïdes* Endl				
Répétitions	3,12	6,25	12,5	T(-)	T(+)	3,12	6,25	12,5	T(-)	T(+)
R1	26,67	26,67	53,34	10	66,67	30	34,34	54,67	5	55
R1	20	20	46,67	10	60	36,67	32	60	0	60
R 3	33,34	33,34	53,34	15	73,34	26,67	35	67	10	80
Moyenne ±σ	26,67 ±6,67 b	37,78 ±3,8 bc	51,11 ±3,85 c	11,66 ±2,88 a	66,67 ±6,7 d	31,11 ±5,09 b	33,78 ±1,58 b	60,56 ±6,12 c	5 ±5 a	65 ±13,23 c

R: Répétitions, T (-): Témoin négatif (PBS : Phosphate-Buffered Saline1X), T(+): témoin positif (Lévamisole à 2,5 mg/ml), σ: Ecart-Type, (a, b, c): différences entre les colonnes pour chaque extrait, les colonnes avec les lettres distinctes ont les moyennes de pourcentage de paralysie larvaire statistiquement différentes entre elles au seuil de 5%.

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Tableau 4: Valeurs des Concentrations entrainants la Paralysie larvaire de 50% des œufs embryonnées obtenues à partir de l'équation de la droite de régression

	R^2 d'ajustement	a	b	CI50
C.sesamoïdesEndl	0,94	0,84	0,09	9,80 mg/ml
S. hermonthica (Deli.) Benth	0,97	0,79	0,07	11,79 mg/ml

a: Ordonnée à l'origine b: pente de la droite régression, CI50: Concentration qui entraine la paralysie larvaire de 50% des œufs embryonnées.

Le tableau 3 nous indique l'effet des extraits aqueux de *S. hermonthica* (Deli.) Benth et de *C. sesamoïdes* Endl sur la paralasie larvaire des œufs embryonnées de *H. contortus*.

Ce tableau nous montre que les extraits aqueux des deux plantes testées ont un taux moyen d'activité de paralysie larvaire des œufs embryonnées de *H. contortus*. En effet, ce tableau nous indique un taux moyen de paralysie larvaire de 51,11% et 60,56% respectivement pour l'extrait aqueux du *S. hermontica* (Deli.) Benth et de celui de *C. sesamoides* Endl.

Nous remarquons un taux de paralysie larvaire de 11, 66% du lot témoin négatif (PBS1X) pour le test avec l'extrait aqueux du *S. hermonthica* (Deli.) Benth et 5% pour le test avec l'extrait aqueux de *C. sesamoides* Endl. Cependant, l'activité du témoin positif (lévamisole) n'a été que de 66,67% et 65% respectivement pour test avec *S. hermonthica* (Del.) Benth et *C. sesamoides* Endl. Le tableau 4 nous indique les valeurs de la droite de régression qui nous a permis d'obtenir les concentrations des extraits des deux plantes testées qui engendrent la paralysie larvaire de 50% des œufs embryonnés.

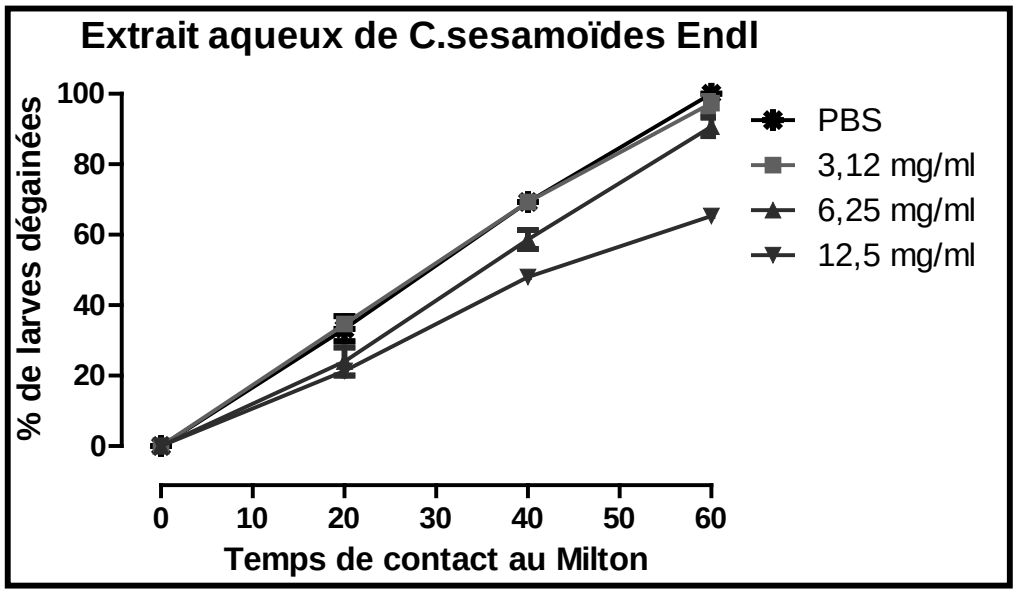


Figure 1: Evolution du dégainement des larves L3 avec l'extrait aqueux de *Ceratotheca sesamoïdes* Endl en fonction du temps

La figure 1 nous montre l'évolution du dégainement en fonction du temps après contact avec la solution de Milton des larves L3 mises en contact avec les différentes concentrations de l'extrait aqueux de *C. sesamoïdes* Endl. La figure 1 nous montre une évolution similaire des courbes du dégainement des larves mises en contact avec le témoin négatif (PBS 1X) et celles mises en contact avec la dose 3,12 mg/ml. L'analyse de ce graphique nous indique aussi que les doses 6,25 mg/ml et 12,5 mg/ml de l'extrait aqueux de *C. sesamoïdes* Endl ont eu une inhibition de dégainement respectivement faible et moyenne des larves.

La comparaison multiple des moyennes du pourcentage de dégainement par la méthode de Tukeys montre une forte significativité entre la moyenne de pourcentage du dégainement des larves L3 au temps (T60) et les temps (T0) ainsi qu'au temps (T20) avec une probabilité $p < 0,001$ alors que la moyenne du pourcentage du dégainement des larves au temps (T60) et celle au temps (T40) n'a pas été très significative $p < 0,011$.

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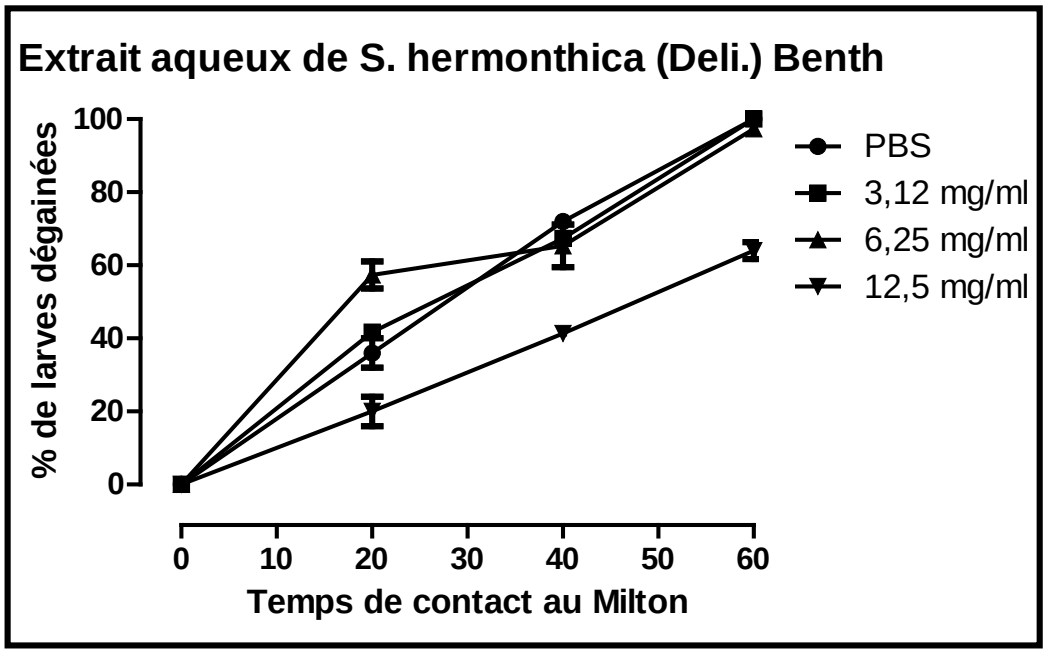


Figure 2: Evolution du dégainement des larves L3 avec l'extrait aqueux de *Striga hermonthica* (Deli.) Benth en fonction du temps

La figure 2 nous montre l'évolution du dégainement des larves L3 en fonction du temps après contact à la solution de Milton des larves L3 mises en contact avec les différentes concentrations de l'extrait aqueux du *S. hermonthica* (Deli) Benth. Ce graphique nous indique une évolution identique des courbes en fonction du temps de la moyenne de dégainement des larves L3 mises en contact avec les concentrations 3, 12 mg/ml, 6,25 mg/ml et celle mises en contact avec le témoin négatif (PBS 1X).

La comparaison multiple des moyennes par la méthode de Tukey montre une forte significativité entre les moyennes du pourcentage de dégainement du temps (T60) et (T20) ainsi qu'aux temps (T60) et temps (T0) $P< 0,001$. Par contre les moyennes du pourcentage du déagienement du temps (T20) et temps (T0) ainsi que temps (T40) et temps (T60) n'ont pas été très significatifs avec respectivement $P< 0,011$ et $P< 0,028$.

IV. Discussion

Les résultats de cette étude nous montrent que l'inhibition de l'éclosion des œufs de *H. contortus* est doses-dépendante avec les extraits aqueux de *C. sesamoïdes* Endl et *S. hermonthica* (Deli.) Benth. En effet, l'extrait aqueux de *C. sesamoïdes* Endl, a obtenu un taux d'inhibition évolutif en fonction de la concentration qui atteint une moyenne de 53,89% à la dose de 12, 5 mg/ml. L'extrait aqueux du *S. hermonthica* (Deli.) Benth a aussi eu un effet inhibiteur évolutif en fonction de la concentration mais de moyenne faible n'atteignant pas 50% à la dose de 12,5 mg/ml. Par contre les extraits des deux plantes testées ont eu un effet inhibiteur et évolutif en fonction de la concentration qui atteint respectivement 51,11% pour *S. hermonthica* (Deli.) Benth et 60,56% pour *C. sesamoïdes* Endl. Koffi et al. (2018), ont eu des résultats similaires avec les extraits hydro-méthanoliques à 2000 µg/ml de sept (07) espèces parmi huit (08) dont seulement l'extraits hydro-méthanoliques du *Morus mesozyna* a eu un de taux d'inhibition de l'éclosion de 84, 08% alors que les sept (07) autres plantes ont eu des taux faibles allant de 5,65% avec *Ficus lutea* à 28, 08% pour *Antiaris africana*. Cette dissemblance de résultats avec l'extrait du *Morus mesozyna* serait due à la nature du solvant d'extraction car les métabolites sécondaire extraits des plantes dépendent du solvant d'extraction. Ainsi, l'extraction aqueuse de nos plantes testées pourrait ne pas contenir les métabolite sécondaire pouvant inhiber l'éclosions des œufs jusqu'à un tel pourcentage que celui du *Morus mesozyna*. Par contre le fait que les sept (07) autres plantes testées par ces auteurs n'ont pas eu 50% d'inhibition de l'éclosions pourrait aussi s'expliquer par la nature leurs solvant d'extractions et aussi par la résistance à des œufs de *H. contortus* qui sont naturellement très résistante. D'Angelo et al. (2014) ont aussi eu des résultats différents au notre avec les extraits methylene Chloro-méthanolique de l'écorcé du tronc de *Annona senegalensis* à différentes doses sur *Heligmosomoïdes Bakeri.* En effet, ces auteurs, ont obtenu un taux d'inhibition de l'éclosion des œufs de 16, 10% alors que le taux de paralysie embryonnaire des œufs embryonnées était de 20,80% à la dose la plus élevée qui est de 5000µg/ml. Cette différence de résultats serait due à la différence de l'espèce de nématode étudiée car notre présente étude porte sur *H. contortus* alors que leur étude à porte sur

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Heligmosoïdes Bakeri. Aussi, cette différence pourrait être due à la différence des plantes utilisées et à la différence des solvants d'extraction qui favorisent la présence de certains métabolites secondaires que d'autres. Les extraits des feuilles de *A. leiocarpus* et des écorces du tronc de *D. oliveri* ont eu un effet dose-dépendant sur la paralysie des œufs embryonnés avec respectivement une CI50 de 411,4 µg/ml pour *A. leiocarpus* et 362,3 µg/ml pour *D. oliveri* et une inhibition de l'éclosion des œufs de 100% pour les extraits aqueux de ces deux plantes (Kaboré, 2009). La différence d'efficacité de ces plantes avec celles des plantes utilisées dans notre présente étude pourrait être due à la nature des plantes utilisées. En effet, notre présente étude a porté sur des herbacées entières alors que cette étude a porté sur des feuilles et écorces de ligneux.

Cette présente étude nous montre à travers les résultats obtenus que les extraits aqueux de *C. sesamoïdes* Endl et ceux du *S. hermonthica* (Deli.) Benth ont une inhibition qui est dose-dépendante sur le dégainèrent des larves L3 de *H. contortus*. En effet, les figure 1 et 2 nous montrent une évolution similaire de la cinétique du dégainement des larves mises en contact avec le témoin négatif (PBS1x) et celles mises en contact avec la dose de 3,12 mg/ml. Par contre l'évolution des courbes des larves mises en contact avec le témoin négatif (PBS1x) et celles des larves mises en contacts avec la dose de 3,12 mg/ml est différente de celle des larves mises en contact avec la doses de 6,25 mg/ml et de celles des larves mise en contact avec la doses de 12, 5 mg/ml dont l'évolution de l'inhibition du dégainement est juste au-dessus de 50%. Les extraits aqueux et méthanolique d'*Acacia nilotica* ainsi que *A. raddiana* ont obtenu une inhibition du dégainement des larves L3 mise en contact avec les doses de 1,2 mg/ml et 0,6 mg/ml qui atteint 100% d'inhibition (Zabré, 2018). La différence avec les résultats obtenus dans cette présente étude pourrait être due à la nature des plantes utilisées car notre présente étude a porté sur les herbacées entières alors qu'il s'agit des feuilles de ligneux dans l'autre étude.

Cette efficacité moyenne des extraits aqueux de *C. sesamoïdes* Endl et *S. hermonthica* (Deli.) Benth serait due à la présence de certains métabolites secondaires tels que les tanins et les flavonoïdes dans ces extraits aqueux. Cependant au vu de résultats d'inhibition de l'éclosion des œufs frais et des œufs embryonnés (paralysie larvaire) ainsi que celui du dégainement des larves L3 nous pouvons émettre l'hypothèse que la solution aqueuse ne permet pas d'extraire la dose suffisante à inhiber l'évolution des œufs et des larves à 100%.

V. Conclusion

La présente étude confirme que les extraits aqueux de *C. sesamoïdes* Endl, et *S. hermonthica* (Deli.) Benth contiennent des composés bioactifs qui inhibe l'évolution des œufs et le dégainement des larves L3 de *H. contortus.* Cependant, du fait que l'inhibition de l'évolution des œufs et du dégainement des larves L3 n'atteint pas 100% nous laisse penser que la présence de ces composés bioactifs serait optimisée par un autre solvant d'extraction. Toutefois les extraits aqueux de *C. sesamoides* Endl et *S. hermonthica* (Deli.) Benth pourraient être employés comme désinfectant sur les pâturages pour interrompre le cycle évolutif des nématodes et diminuer ainsi la charge parasitaire des pâturages.

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Supercritical Fluids as a State-of-the-Art Formulation Method of Nanoparticles for Ocular Drug Delivery

By Naida Omerović & Edina Vranić

University of Sarajevo

Abstract- Conventional ophthalmic dosage forms, although being simple to apply and presenting great patients' compliance, display poorer drug bioavailability and retention time on the eye surface. To cope with these problems, one must formulate novel drug delivery systems, such as nanosystems, for ocular drug delivery. Different formulation methods of nanoparticles have been developed, but some of them, such as the supercritical fluid method, have not reached their full potential in ocular drug delivery. This article aims to present the possibilities of the supercritical fluid method when preparing nanosystems for ocular drug delivery. This method could be used more frequently and efficiently because it is environmentally friendly and produces nanoparticles of the desired physicochemical properties, which is especially important in ocular drug delivery considering its peculiarities. Modifications of the supercritical fluid method can be used when a drug has some specific properties, which is an additional benefit in ocular drug delivery.

Keywords: *nanosystems, nanoparticles, ocular drug delivery, supercritical fluids.*

GJMR-B Classification: *NLMC Code: QV 785*

SUPERCRITICALFLUIDSASASTATEOFTHEARTFORMULATIONMETHODOFNANOPARTICLESFORDRUGDELIVERY

Strictly as per the compliance and regulations of:

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Supercritical Fluids as a State-of-the-Art Formulation Method of Nanoparticles for Ocular Drug Delivery

Supercritical Fluids in Nano-Ophthalmology

Naida Omerović [α] & Edina Vranić [σ]

Abstract- Conventional ophthalmic dosage forms, although being simple to apply and presenting great patients' compliance, display poorer drug bioavailability and retention time on the eye surface. To cope with these problems, one must formulate novel drug delivery systems, such as nanosystems, for ocular drug delivery. Different formulation methods of nanoparticles have been developed, but some of them, such as the supercritical fluid method, have not reached their full potential in ocular drug delivery. This article aims to present the possibilities of the supercritical fluid method when preparing nanosystems for ocular drug delivery. This method could be used more frequently and efficiently because it is environmentally friendly and produces nanoparticles of the desired physicochemical properties, which is especially important in ocular drug delivery considering its peculiarities. Modifications of the supercritical fluid method can be used when a drug has some specific properties, which is an additional benefit in ocular drug delivery.

Keywords: nanosystems, nanoparticles, ocular drug delivery, supercritical fluids.

I. Introduction

Different eye barriers present a challenge for drug delivery to the eye (Figure 1). These barriers are [1,2]:

- Anatomical (conjunctiva, sclera, choroid, retina, with the blood-retinal barrier (BRB) comprising retinal capillary endothelial cells and retinal pigment epithelium (RPE)).
- Physiological (removal of the solution by blinking, dilution of the solution by tearing).

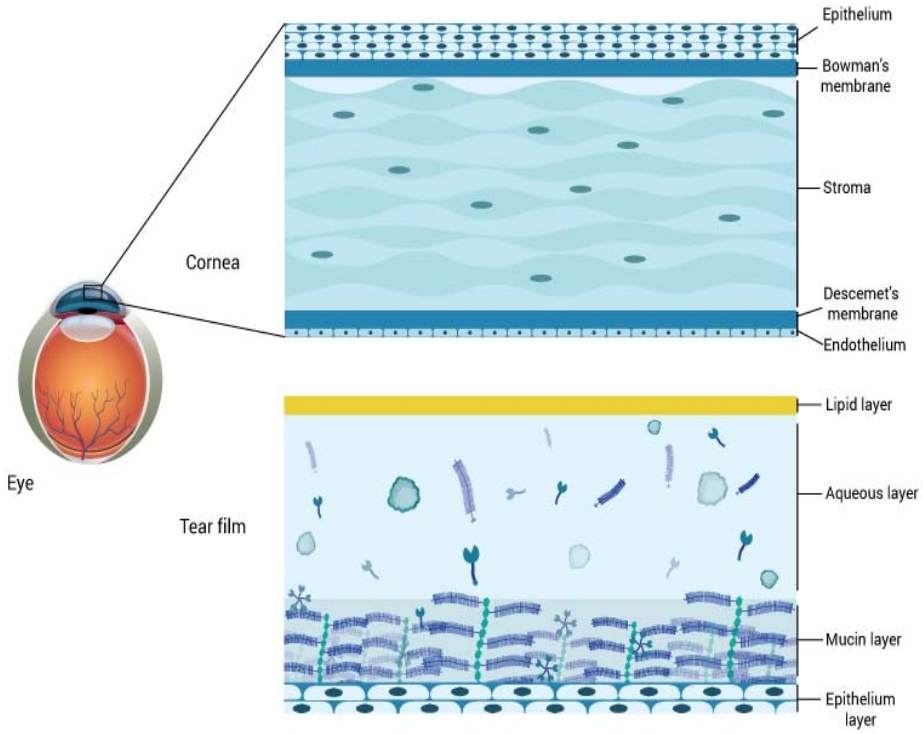


Figure 1: Different eye layers

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They are an obstacle during the development of different ophthalmic dosage forms [3,4]. Conventional ophthalmic dosage forms include eye drops, ointments, suspensions, and emulsions. These formulations have proven their advantages, such as great patients' compliance and easy applicability, but their limitations include more frequent administration to get the therapeutic drug concentration, as well as poor drug bioavailability. Conventional ophthalmic dosage forms are more powerful in the treatment of the diseases of anterior rather than posterior eye segment. Previously mentioned dosage forms can also cause the occurrence of eye irritation, eye redness, and visual impairment [5,6].

To cope with these disadvantages of conventional ophthalmic dosage forms, novel drug delivery systems for ocular drug delivery are being developed, wherein nanosystems get special attention. Nanosystems that can be used as ocular drug delivery systems include nanoparticles, nanomicelles, liposomes, niosomes, dendrimers, nanosuspensions, nanoemulsions, nanocrystals, etc (Figure 2). Their advantages are [7,8]:

- Higher: drug permeability, bioavailability, and retention on the eye surface
- Lower: drug degradation, risk of visual impairment, required drug doses, and occurrence of side effects.

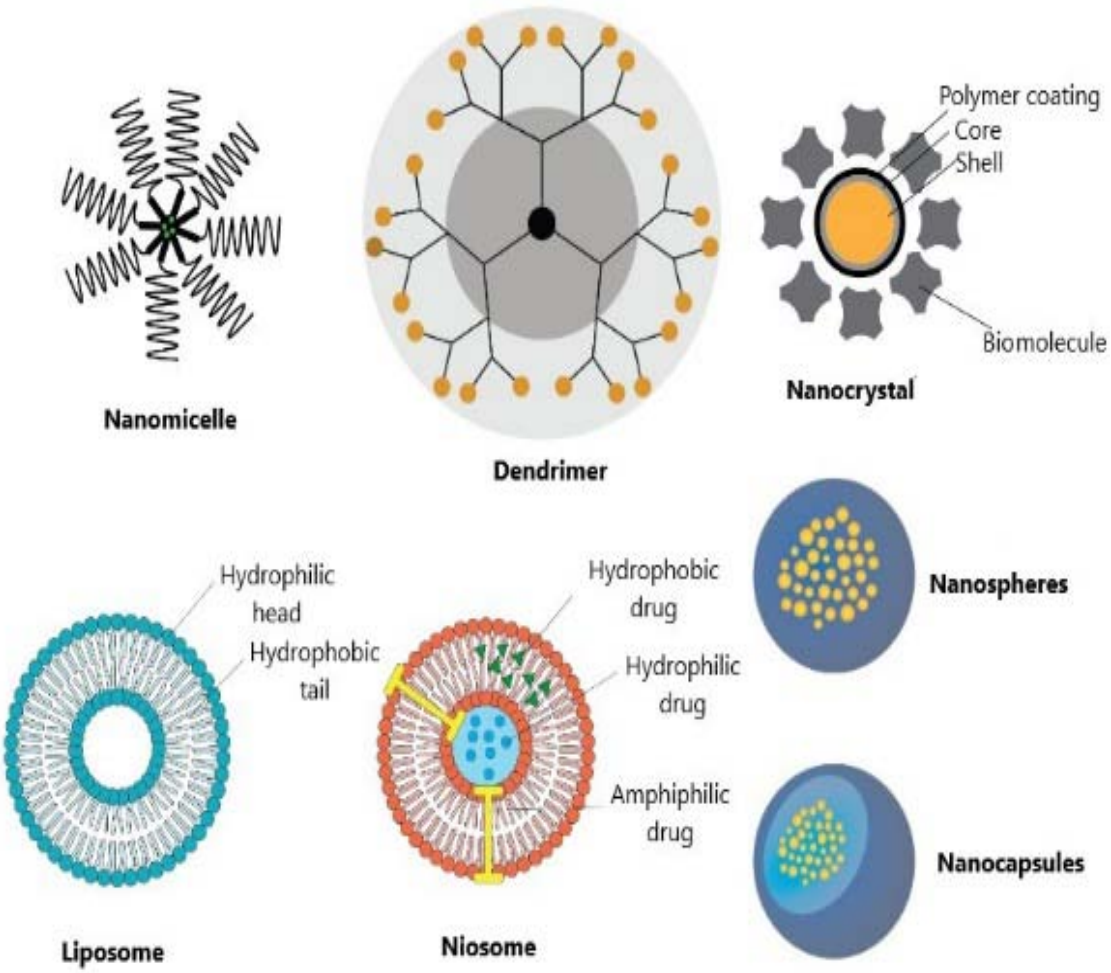


Figure 2: Types of nanosystems

There are two classifications of nanoparticles, the first being nanospheres and nanocapsules, and the second being polymeric nanoparticles and lipid nanoparticles. Nanospheres are matrix systems, in which a drug is adsorbed on the surface or evenly dispersed in the matrix. They have a solid core with a dense polymer around it, with a size from 100 to 1000 nm. Nanocapsules are vesicular systems, in which a drug is usually dissolved in the particle's nucleus, but it can also be adsorbed on the surface. They have an oil-filled cavity with a firm polymeric envelope around it, with a size from 10 to 1000 nm [9,10]. In polymeric nanoparticles, a drug is dissolved or encapsulated in different polymers. These nanoparticles have a size from

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10 to 100 nm, are biodegradable, non-toxic, biocompatible, and mucoadhesive. Polymers can be made either by the direct process of polymerisation of different monomers or from derived polymers [11]. Lipid nanoparticles comprise lipids that are solid at room and body temperature, unlike water/oil (w/o) emulsions that have liquid lipids. These nanoparticles have a size from 150 to 300 nm, are non-toxic and long-term stable, protect a drug from its degradation, present controlled drug release and improved drug bioavailability. There are two groups of lipid nanoparticles: solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) [12]. The SLN comprise a solid lipid with an incorporated drug, and water, co-emulsifiers, and emulsifiers as excipients [13]. The NLC incorporate liquid and solid lipids as the basic matrix [14].

Factors that must be considered when designing an ocular drug delivery nanosystem are [15]:

- Size of nanoparticles
- Blood and lymphatic circulations
- Melanin pigmentation
- Diseases
- Functionalization of nanoparticles
- Charge of nanoparticles.

These factors are discussed in the following paragraphs.

Amrite et al. conducted a study to determine the distribution of carboxylate-modified, negatively charged polystyrene (PS) nanoparticles in the eye. Nanoparticles, sizes of 20 nm and 200 nm, were applied subconjunctivally to Sprague Dawley rats. Nanoparticles of 200 nm were detected two months post-application in the periocular tissue of the eye, whereas nanoparticles of 20 nm were not, as they were fastly eliminated from that tissue. Subconjunctivally applied nanoparticles of 200 nm and larger could be kept at the site of application for at least two months, which meant that nanosystems of this size could be useful as controlled drug delivery nanosystems for periocular application [16]. Amrite et al. conducted another study to investigate the transscleral permeability of celecoxib-loaded nanoparticles, sizes of 20 nm and 200 nm, in the presence and absence of blood and lymphatic circulations in Sprague Dawley rats. Various clearance rates showed that the clearance of nanoparticles affected the concentrations of celecoxib in the retina. Larger nanoparticles showed sustained drug delivery in the retina better compared with the smaller ones, but these differences were reduced with an increase in the release rate of celecoxib from nanoparticles. Blood and lymphatic circulations played an important role in the clearance of the 20 nm-sized nanoparticles, which were transported across the sclera in a minimal amount, but with significant transport across the sclera-choroid-RPE, because of their fast disposition in the presence of blood and lymphatic circulations. The 200 nm-sized nanoparticles displayed low clearance by blood and lymphatic circulations and were suitable for controlled transscleral drug delivery [17].

Cheruvu et al. conducted a study to determine the effect of the melanin pigmentation on the transscleral delivery of celecoxib-loaded nanoparticles in Sprague Dawley (non-pigmented, albino) and Brown Norway (pigmented) rats. The affinity and the extent of the binding of celecoxib to natural melanin were not significantly different compared with those to synthetic melanin. Transscleral retinal and vitreal drug deliveries of celecoxib were significantly lower in Brown Norway rats compared with those in Sprague Dawley rats, which could be attributed to the significant binding of celecoxib to melanin and its accumulationin the melanin-rich choroid-RPE [18]. Cheruvu et al. conducted another study to investigate the effect of diabetes on the transscleral delivery of celecoxib-loaded nanoparticles in Sprague Dawley and Brown Norway rats. Transscleral retinal and vitreal drug deliveries were significantly higher in diabetic animals of both Sprague Dawley and Brown Norway rats, which could be attributed to the disruption of the BRB because of diabetes. Transscleral delivery of celecoxib was higher in Brown Norway rats compared with that in Sprague Dawley rats [19].

Kompella et al. conducted a study to determine whether topical ocular drug delivery of nanoparticles of size <100 nm could be enhanced by their coating with peptides or proteins in the bovine eye. The uptake of nanoparticles was in the following order: corneal epithelium>stroma>endothelium, with concentrations in the aqueous humour being undetectable. Transport across the cornea was enhanced because of the functionalization of PS nanoparticles of 20 nm with deslorelin, a luteinizing hormone-releasing hormone (LHRH) agonist, as well as transferrin. Surface modification of nanoparticles by conjugating a LHRH agonist or transferrin was useful to provide fast and efficient delivery of nanoparticles across the cornea [20]. Eljarrat-Binstock et al. conducted a study to determine the penetration of positively and negatively charged fluorescent nanoparticles in rabbits. Positively charged nanoparticles showed better penetration into the inner ocular tissues compared with the negatively charged ones [21]. Kim et al. conducted a study to investigate the movement of intravitreally applied human serum albumin (HSA) nanoparticles concerning the surface charge of nanoparticles. Either cationic or anionic HSA nanoparticles were injected to determine the effect of surface charge on the intravitreal movement of nanoparticles. Anionic nanoparticles with a surface charge of -33.3 ± 6.1 mV diffused more easily through the collagen fibres compared with the cationic ones with a surface charge of 11.7 ± 7.2 mV. Anionic HSA nanoparticles presented a promising drug delivery nanosystem to the subretinal ocular tissue and RPE

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[22]. Cationic polymers highly interact with the negatively charged hyaluronan in the vitreous cavity of the eye. The PS nanoparticles showed an interaction with collagen fibres in the sclera, which resulted in their poor diffusion through the vitreous cavity. Many modifications have been developed to cope with the effect of the above-mentioned factors, such as [23]:

- Surface modification of nanoparticles with poly(ethylene glycol) (PEG), also known as PEGylation
- Masking the reactive surface of nanoparticles
- Targeting the specific transporters or receptors on the cell surface.

Having in mind all these specificities of ocular drug delivery, as well as the fact that newer formulation methods of nanoparticles, such as the supercritical fluid method, have not been used in preparing ocular drug delivery nanosystems so much, this article aims to present the possibilities of the use of the supercritical fluid method when preparing nanosystems for ocular drug delivery.

II. Formulation Methods of Nanoparticles for Ocular drug Delivery: Classification and Steps

The most important methods of formulating the nanoparticles that are used in ocular drug delivery can be classified as [24,25]:

- Polymerisation of a monomer:
  - o Emulsion polymerisation
  - o Interfacial polymerisation
  - o Interfacial polycondensation
- Production from pre-formed polymers:
  - o Solvent removal
  - o Interfacial deposition
  - o Emulsification – solvent evaporation
  - o Emulsification – solvent diffusion
  - o Salting-out
- Production from natural macromolecules:
  - o Ionic gelation
- Desolvation of macromolecules
- Newer methods: supercritical fluid
- Other methods: homogenisation and milling.

a) *Supercritical fluid*

A supercritical fluid is a substance under a condition above its critical points, which are the highest temperature and pressure at which a substance exists as the gas and the liquid in equilibrium. Thus, a supercritical fluid shows the properties of both gases (such as penetration) and liquids (such as dissolution) simultaneously. Gases, such as carbon dioxide (CO_2), ammonia (NH_3), or water (H_2O), can be converted to supercritical fluids under particular conditions. But only supercritical CO_2 can be economically useful because its critical points (temperature = 31.1 °C, pressure = 73.8 bar) are relatively easy to reach [26].

Supercritical fluids have great potential in the production of nanosystems but have not been used frequently in ocular drug delivery. Studies conducted so far include the preparation of dexamethasone [27] and griseofulvin [28] nanoparticles. This method can replace the use of organic solvents while preparing nanoparticles for advanced drug delivery and formulation systems, such as the ocular ones. What makes a difference between conventional formulation methods, such as freeze- or spray-drying, where larger particles are primarily formed and then reduced to the desired size, is the fact that the supercritical fluid method instantly forms particles in a manner to get the desired properties. This is the way to avoid the unwanted effects caused by the energy transfer in the system, which is needed to reduce the size of particles. Particles formed this way do not have to undergo additional treatment, which is a significant advantage [29,30].

The most important characteristic of this method is the fact that there is no phase boundary between the gas and the liquid, so there is continuity in the physicochemical properties of the fluid. With small variations in pressure and temperature of the system, the properties of supercritical fluids are alterable from the gas to the liquid, therefore the viscosity and diffusivity are closer to those of the gas and the density is closer to that of the liquid [31].

The most important types of the supercritical fluid method include [24,32]:

- Supercritical anti-solvent (SAS)
- Rapid expansion of the supercritical solution (RESS).

i. *Supercritical anti-solvent (SAS)*

The SAS method includes the precipitation of a solute in a compressed fluid under supercritical conditions. The precipitation can be done in two ways [33]:

1. An anti-solvent can be added to a solution (normal-addition precipitation)
2. A solution can be added to an anti-solvent (reverse-addition precipitation).

A supercritical anti-solvent must be miscible with a solution solvent, whereas a solute must not be soluble in a supercritical anti-solvent [33].

In normal-addition precipitation, first, a solute is dissolved in a liquid solvent, and second, a supercritical anti-solvent is added to a solution. The latter is done in a partially filled container at the ambient pressure. With the addition of a supercritical anti-solvent, both the pressure of a closed container and the volume of a solution/anti-solvent mixture increase. With an increase in the anti-

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solvent fraction in the mixture and a decrease in solubility of a solute, the precipitation of a solute occurs. To get nanoparticles of the desired physicochemical properties, the precipitate is washed with an anti-solvent [33].

In reverse-addition precipitation, first, a liquid solution is sprayed into a supercritical anti-solvent. The precipitation of a solute occurs because of the fast diffusion of a solvent from the solution sprayed into a supercritical anti-solvent. Second, the precipitate is washed with an anti-solvent and filtered to get nanoparticles of the desired physicochemical properties [33].

No matter whether the precipitation of a solute is normal or reverse, both the size and size distribution of nanoparticles depend on [33]:

- The selection of a solution/anti-solvent mixture
- The concentration of a solute
- The relative quantities of a solution and an anti-solvent
- The rate of the anti-solvent addition
- The degree of mixing.

In a study conducted by Subramaniam et al., the size of nanoparticles depended on the pressure because smaller nanoparticles are formed when the pressure of an added anti-solvent increases. This was attributed to the faster nucleation of a solute [34]. In a study conducted by Rantakylä et al., the size of nanoparticles depended on both temperature and pressure, but was independent of the nozzle exit velocity and diameter. The initial size of droplets did not affect the size of nanoparticles [35].

The SAS method is presented in Figure 3.

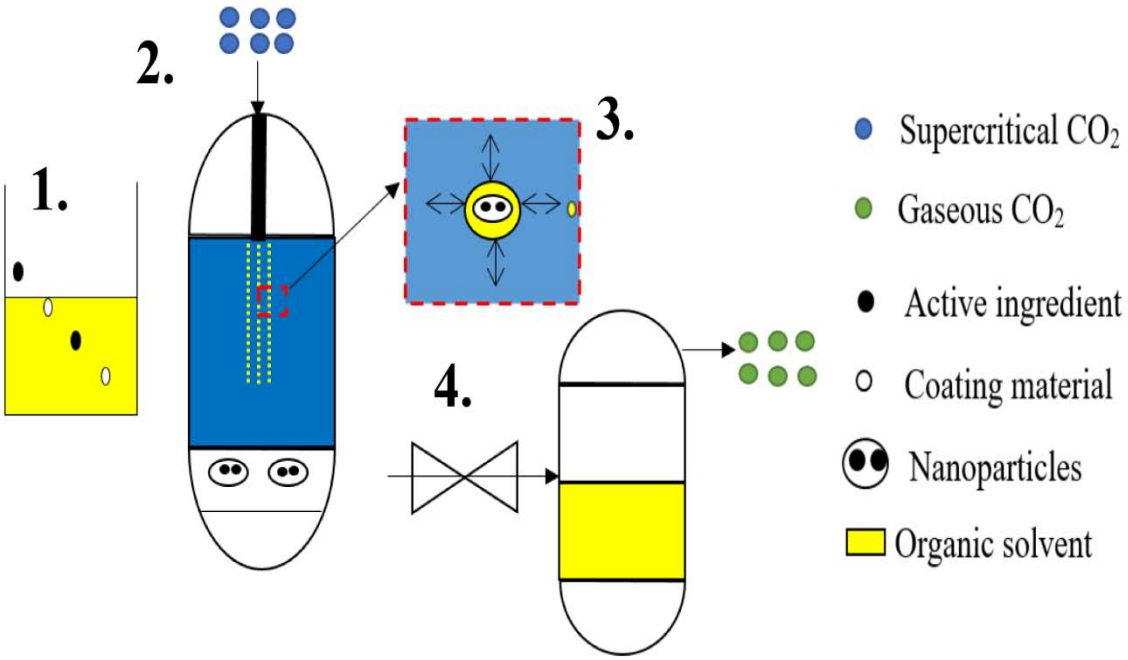


Figure 3: The supercritical anti-solvent (SAS) method

1. An active ingredient and a coating material are dissolved in an organic solvent.
2. The organic solution is sprayed into supercritical CO_2.
3. Supercritical CO_2, as an anti-solvent, leads to supersaturation and precipitation of nanoparticles.
4. When the expansion occurs, CO_2 reverts to its gaseous state, while an organic solvent is being removed.

One advantage of the SAS method is the fact that supercritical fluids are environmentally safe, thus this method could be used for preparing biodegradable nanoparticles, which is recommended in ocular drug delivery. Nevertheless, there are some new modifications of this method, which include the nebulisation of a solution by supercritical fluids that also behave as anti-solvents to precipitate a solute. Here, a solution and a supercritical fluid are mixed and sprayed into a drying atmosphere. Examples are [36–39]:

- CO_2-assisted nebulisation with a bubble dryer (CAN-BD)
- Supercritical fluid-assisted atomisation (SAA).

A big disadvantage of the SAS method is the fact that the formation of nanoparticles is followed by a long drying period, which can lead to their agglomeration and aggregation. Still, this problem is manageable by intensive mixing of a solution with a supercritical anti-solvent. This can lead to a more efficient and enhanced mass transfer caused by the sonic waves in an energising gas stream, as well as the smaller size of droplets, by using an ultrasonic nozzle. The preparation of nanoparticles at low temperature is

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important for drugs that are thermally labile or shock-sensitive, such as some drugs used in the treatment of ocular diseases. Still, many substances have a higher solubility in liquid solvents compared with low-temperature supercritical fluids, and the SAS method allows a higher flow rate [40].

ii. *Rapid expansion of the supercritical solution (RESS)*

The RESS method differs from the SAS method because, in the RESS method, first, a solute is dissolved in a supercritical fluid to form a solution, and second, a solution is quickly expanded into an area of lower pressure or ambient air [41–43]. A rapid decrease in pressure and density causes the precipitation of a solute. A supercritical solution can be generated in two ways, by [44]:

- Heating and pressurizing a solution at room temperature (expansion is performed at a constant concentration)
- Continuously extracting a solute with an extraction column (useful for solutes insoluble in solvents).

The temperature of a solvent can differ from or be the same as the temperature at which the rapid expansion is performed. Both the extraction temperature and flow rate control the concentration of a solute. Rapid expansion from supercritical to ambient pressure leads to supersaturation because the solubility of supercritical fluids can be over 100 times higher compared with that under ideal gas conditions. Fast reduction of pressure results in the homogeneous nucleation of a solute but narrow size distribution of nanoparticles [44].

The RASS method can be used for preparing organic, inorganic, or polymeric nanomaterials. Fibres of polymeric nanomaterials can be prepared under the controlled expansion conditions, therefore variations in these conditions allow modifications of physicochemical properties of nanoparticles. Pre-expansion temperatures that are significantly higher or lower compared with the melting point of a polymer are used to create particles, whereas those that are close to the melting point of a polymer are used to create fibres [44].

The RASS method could also create nanoparticles with the controlled release of drugs, which could be very useful in ocular drug delivery [29]. The physicochemical properties of nanoparticles depend on various processing conditions, which allow control over them. Microparticles are generally obtained as primary products, but nanoparticles (100–300 nm) can also be produced in the RESS method by using the appropriate nozzles [45].

The RESS method requires minimum to no organic solvent. The precipitation process is enhanced because of the decrease in pressure and temperature that a solute goes through during the rapid expansion [46]. Caution should be taken regarding the occurrence of agglomeration and aggregation during the rapid expansion [47,48].

A big disadvantage of the RESS method with supercritical CO_2 is the low solubility of polar drugs. This method only applies to solutes that show good solubility in supercritical CO_2. Poorly soluble substances with high molecular weights and polar bonds are great candidates for preparing nanoparticles, but many of them have low to negligible solubility in supercritical CO_2 at temperatures less than 60 °C and pressures less than 300 bar. Co-solvents could be added to CO_2 to enhance drug solubility [46]. To cope with this problem, a modified method of RESS with solid co-solvent (RESS-SC) was proposed. A solid co-solvent provides an obstacle for coagulation, enhances the solubility, and can be later removed by lyophilisation (sublimation) [49–51]. Rapid expansion from supercritical to aqueous solution (RESAS) is another modification, which produces nanoparticles of water-insoluble drugs [52–54].

The RESS method is presented in Figure 4.

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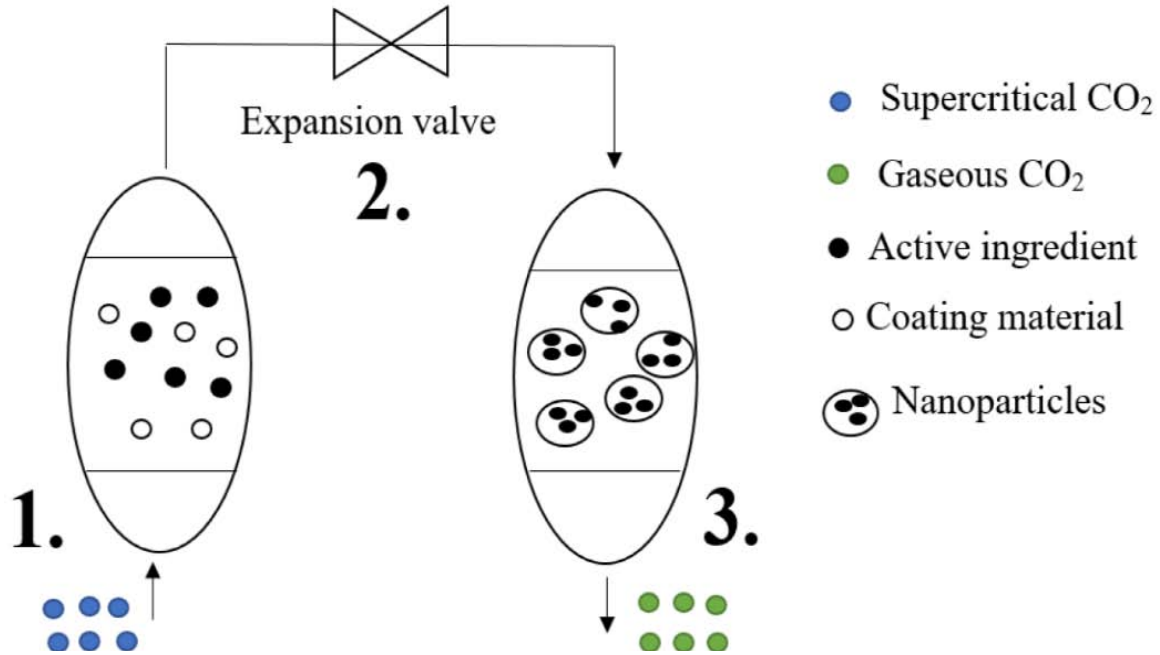


Figure 4: The rapid expansion of the supercritical solution (RESS)

1. An active ingredient and a coating material are dissolved in supercritical CO_2.
2. When the expansion occurs, CO_2 reverts to its gaseous state.
3. Nanoparticles are formed because of the decrease in solubility and precipitation of an active ingredient and a coating material.

iii. *Operating parameters affecting the properties of nanoparticles formed by the supercritical fluid method*

Various thermodynamic and aerodynamic factors, as well as properties of the material, affect the morphology, shape, size, and size distribution of nanoparticles. Thermodynamic factors include temperature, pressure, rate of addition of substances, as well as phase and composition changes during the expansion. Aerodynamic factors include nozzle geometry, mechanical shear, and impact distance of the jet against a surface [46].

The supercritical fluid method allows the production of tiny particles (such as nanoparticles) and experiments conducted so far have shown that the size of particles, as well as some other properties, depend on a solvent, a solute, and the pre-expansion conditions. Still, there are some dilemmas regarding the relationship between the conditions of the process and the mechanism of the formation of particles. The interaction between nucleation, condensation, and coagulation is also important to understand. Thus, there is a need to model the supercritical fluid method and its conditions to better understand how different operating parameters affect the properties of the final product, such as nanoparticles. Some of the most cited studies conducted regarding the operating parameters affecting the properties of nanoparticles formed by the supercritical fluid method are presented in Table 1, along with their most important remarks.

Table 1: Studies conducted regarding the operating parameters affecting the properties of nanoparticles formed by the supercritical fluid method

STUDY	REMARKS
[55]	Mathematical modelling of particle growth and nucleation during the supercritical fluid method was presented. The flow rate was steady and one-dimensional. Partial expansion of supercritical solutions was proven to be an effective way to form powders of desired size and other properties. The size of particles responded to the extraction temperature (the temperature at which a solute is dissolved in a supercritical fluid), as well as the pre-expansion temperature (the temperature at which the saturated mixture is isobarically pre-heated before the expansion). A decrease in solubility upon isobaric heating embodied three trends predicted by calculations: an increase in the size of particles upon the increase in pre-expansion temperature and a decrease in the size of particles upon the increase in extraction temperature and pressure.
[56]	Hydrodynamic modelling of the RESS method was presented. The expansion occurred at the nozzle inlet, along with the nozzle, and in the expansion chamber, and the thermodynamic transformations for each step were calculated, along with the temperature and pressure profiles. Flow rate at the nozzle exit was measured for two supercritical fluids, CO_2 and fluoroform (CHF_3), at different pre-expansion temperatures and pressures. Computed values were comparable with the experimental data.

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[57]	A one-dimensional flow modelling of the RESS method was presented. The emphasis was on the wall friction in the nozzle and heat interchange with the surrounding region. Calculations were performed on the ibuprofen–supercritical CO_2 system and supersaturation, as well as nucleation, were considered. Results showed that very high supersaturation and nucleation rates can be obtained when the fluid leaves the nozzle exit. A decrease in the size of particles was also visible. Sensitivity analyses showed that pre-expansion temperatures and pressures, as well as the nozzle length, did not significantly influence the flow rate.
[58]	Modelling of the thermodynamic behaviour of supercritical solutions during the RESS method was presented and an impact of the solubility and surface tension of different solutes in supercritical fluids on nucleation rates under typical RESS conditions was investigated. Calculations showed that the nucleation rate is a solubility and surface tension function. It was shown that it is not possible to investigate coagulation and homogeneous nucleation separately. Also, the information about the properties of a solute needs to be more reliable.
[59]	Experiments were carried out with CO_2 and CHF_3 as supercritical fluids, as well as with cholesterol, benzoic acid, and griseofulvin. It was shown that it is possible to produce particles of the desired size, which leads to improved dissolution. Flow rate and the formation and growth of particles during the RESS method were modelled numerically. The flow rate was steady and one-dimensional. Results showed that the formation of particles mainly occurs in the supersonic free jet and that the primary mechanism of particle growth is coagulation.
[60]	The size distribution of fine powders formed during the RESS method depends on the operating parameters and the geometry of the expansion utensil. Homogeneous nucleation, condensation, and coagulation during the expansion of a non-volatile solute in a supercritical fluid were modelled. Calculations showed that the RESS method is very successful in the formation of particles in the diameter range of 10–50 nm. Delayed nucleation, precipitation of a small solute fraction, and narrow size distribution of particles were mostly displayed.
[61]	In this study, it was shown that the aerodynamic factors remain constant over a wide range of pressures. The formation of particles was probably a result of gas-phase nucleation and growth within the expansion utensil, rather than within discrete liquid droplets.
[62]	In this study with the toluene (solvent)–CO_2 (anti-solvent) system, it was displayed that the size of droplets depends on densities of both solvent and anti-solvent. It is recommended to work in a single-phase zone at high pressure because higher mass transfer and supersaturation ratio are then achieved. Mathematical modelling of mass transfer between an organic solvent and an anti-solvent was presented when two phases were miscible. The mass transfer behaviour of droplets could affect the morphology of particles. Under supercritical conditions of the mixture, a solvent and an anti-solvent were miscible. Thus, the interface between the droplets and their environment was not defined. By defining a radius of droplets based on the difference in density between a solvent and an anti-solvent, the mass transfer can be calculated, as well as an impact of the operating parameters. Calculations showed droplets swell when a solvent has a higher density compared with an anti-solvent, and shrink when an anti-solvent has a higher density compared with a solvent. Near the critical points of the mixture, droplets swelled and were more sensitive to changes in temperature and pressure. The lifetime of droplets was significantly shorter under miscible conditions. When the critical points of the mixture are close, droplets swelled less. The extent of droplet swelling or shrinking was a temperature and pressure function correlated to the difference in density and diffusivity between a solvent and an anti-solvent. Supercritical conditions resulted in faster mass transfer, causing higher supersaturation and nucleation rates but the smaller size of particles.
[63,64]	The authors of these two studies investigated whether the spherical shape of particles results from the drying of droplets after the atomisation of a liquid solution. Above the critical pressure, the surface tension influences the formation of particles. When atomisation is not obtained, the precipitation from the fluid phase produces particles. They also investigated the relationship between the solute concentration, the vessel temperature and pressure, as well as the fraction of CO_2. They concluded that the fraction of CO_2 greater than 0.95–0.97 is the most useful for the formation of smaller particles. Critical points of the mixture of CO_2 and a liquid organic solvent were then achieved.

III. Conclusion

Novel drug delivery systems are continuously being developed for ocular drug delivery because of their many advantages compared with conventional ophthalmic dosage forms. Nanoparticles have especially been receiving attention. The most important formulation methods of nanoparticles for ocular drug delivery include polymerisation of a monomer (emulsion polymerisation, interfacial polymerisation, interfacial polycondensation), production from pre-formed polymers (solvent removal, interfacial deposition, emulsification – solvent evaporation, emulsification – solvent diffusion, salting-out), production from natural macromolecules (ionic gelation), desolvation of macromolecules, newer formulation methods (supercritical fluid), and other formulation methods (homogenisation, milling). The choice of the formulation method depends on an active pharmaceutical ingredient and other substances used, as well as the desired physicochemical properties of nanoparticles. New formulation methods are constantly being developed to get the most out of nanotechnology, such as the supercritical fluid method. The SAS method uses a liquid solvent and a supercritical fluid, which are miscible with each other, but a solute is not soluble in a

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supercritical fluid. Instantaneous precipitation of a solute, which is formed because of the extraction of a liquid solvent by a supercritical fluid, causes the formation of nanoparticles. In the RESS method, a supercritical fluid dissolves a solute. Consequently, the solvent power of a supercritical fluid is reduced and a solute precipitates because of its rapid expansion into an area of lower pressure. These methods can create nanoparticles of adequate size, size distribution, and other properties for ocular drug delivery, and should be applied more regularly.

Conflict of interest

The authors declare that there was no conflict of interest that could be perceived as prejudicing the impartiality of this research.

Originality

The authors confirm that this manuscript is their original work and has not been previously published nor is sent to another journal for consideration.

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Authors' contributions

Naida Omerović: concept/design, data collection, data analysis/interpretation, drafting the manuscript, critical revision of the manuscript, approval of the manuscript; Edina Vranić: concept/design, data analysis/interpretation, critical revision of the manuscript, approval of the manuscript.

Consent

Not applicable.

Ethical approval

Not applicable.

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Abbreviations

BRB – blood-retinal barrier

CAN-BD – CO2-assisted nebulisation with a bubble dryer

HSA – human serum albumin

LHRH – luteinizing hormone-releasing hormone

NLC – nanostructured lipid carriers

PEG – poly(ethylene glycol)

PS – polystyrene

RESAS – rapid expansion from supercritical to aqueous solution

RESS – rapid expansion of the supercritical solution

RESS-SC – rapid expansion of the supercritical solution with solid co-solvent

RPE – retinal pigment epithelium

SAA – supercritical fluid-assisted atomisation

SAS – supercritical anti-solvent

SLN – solid lipid nanoparticles

w/o – water/oil

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The Knowledge, Attitude and Practice of Taking Covid-19 Vaccination among the Rural and Urban People in Patuakhali District

By Sultan Ahmed Sikder, Sharmin Jahan Shubarna, Sahedul Islam Bhuiyan & Mohoshina Karim

Abstract- *Objective:* In this study our main goal is to evaluate the Knowledge, Attitude and Practice of taking Covid-19 vaccination among the rural and urban people in Patuakhali District.

Method: This cross-sectional study was carried out at Tertiary medical College Hospital, Patuakhali District. Bangladesh. Where data were collected from July 2020 to May 2021. A total of 120 participants where in rural 60 participants and in urban 60 participants were included in the study.

Results: In this study total respondents were 120. In rural group highest 23 (38.33%) respondents belong to 32-43 years and in urban group highest 31 (51.66%) respondents belong to 44-55 years. Most of the respondents were male. In rural area highest 30 (50%) respondents were HSC passed followed by primary 13 (21.7%) and in urban area highest 25 (41.25%) respondents were HSC followed by graduate 18 (30%).

Keywords: *Covid-19, vaccine, KAP study.*

GJMR-B Classification: *NLMC Code: WA 115*



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The Knowledge, Attitude and Practice of Taking Covid-19 Vaccination among the Rural and Urban People in Patuakhali District

Sultan Ahmed Sikder [α], Sharmin Jahan Shubarna [σ], Sahedul Islam Bhuiyan [ρ] & Mohoshina Karim [ω]

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Results: In this study total respondents were 120. In rural group highest 23 (38.33%) respondents belong to 32-43 years and in urban group highest 31 (51.66%) respondents belong to 44-55 years. Most of the respondents were male. In rural area highest 30 (50%) respondents were HSC passed followed by primary 13 (21.7%) and in urban area highest 25 (41.25%) respondents were HSC followed by graduate 18 (30%). The association of Covid-19 infected status between rural & urban respondents were highly significant (P= 0.00), similarly knowledge regarding first vaccine in Bangladesh also highly significant (p=0.02) between rural & urban respondents. Another findings regarding knowledge related factor regarding the lowest age range for this vaccination was moderately significant (p=0.03), finally knowledge related factor regarding the way to take the vaccine was also significantly associated (p=0.04) between the respondents of rural & urban area, beside the knowledge related factors the other variables were not significantly associated. The association of Covid-19 vaccine taken status between rural & urban respondents were highly significant (P= 0.00), similarly the opinion regarding so many rumors about corona vaccine also moderately significant (p=0.03) others attitude and practice related factors were not significantly associated.

Conclusion: The COVID-19 pandemic is still experiencing worldwide disasters and lives, but a possible ray of hope for the future can be found with the COVID-19 vaccine. The findings recommend immediate programs of health education and that the respective health authorities should provide more accurate information. In order to decrease vaccine relief enabled and promoted by disinformation in the media, policymakers should take efforts to provide appropriate understanding, favorable attitudes and views of COVID-19 immunization.

Keywords: Covid-19, vaccine, KAP study.

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I. Introduction

As SARS-CoV-2 is highly infectious virus that impacts communities around the globe, vaccines are the most significant measure of public health and the most effective approach for protecting the population against COVID-19. The competitive effort for the discovery and development of COVID-19 vaccines against the spread and disastrous consequences of the illness is ongoing with the creation of new, more effective vaccinations as the pandemic passes. [1-2]The distribution of vaccines is in the process and the acceptance of COVID-19 immunizations by the community must be investigated. [3]

On 27 January 2021 the Bangladeshi authorities decided to utilize the Indian vaccination Covidshield; Runu (A Nurse) was the first COVID-19 receiver. [4]

Bangladeshi officials approved. However, there is a large debate among the general people of Bangladesh over COVID-19 vaccines. A fraction of the people of Bangladesh hesitate to take the Indian vaccination so that they are not infected. [5] A worldwide COVID-19 research showed that 48 percent of the study population had misunderstandings with the COVID-19 vaccines and were doubtful about their vaccination. [6]

In this study our main goal is to evaluate the Knowledge, Attitude and Practice of taking Covid-19 vaccination among the rural and urban people in Patuakhali District.

II. Objective

- To assess the Knowledge, Attitude and Practice of taking Covid-19 vaccination among the rural and urban people.

III. Methodology

a) Types of study

- It was a cross sectional study.

b) Place and period of the study

- The study place was carried out at Tertiary medical College Hospital, Patuakhali District. Bangladesh. Where data were collected from July 2020 to May 2021.

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c) *Study population*

- A total of 120 participants where in rural 60 participants and in urban 60 participants were included in the study. Sample were collected through purposive sampling as per inclusion criteria.

d) *Method*

- Data were collected by using a pre designed questionnaire. The questionnaire was prepared reviewing literature and consulting with medical research experts.

e) *Data analysis*

- All collected data were coding and input in SPSS-25 for further analysis. Both descriptive and inferential statistics done. Descriptive statistics included frequency distribution, percent, mean, standard deviation; graph, tables, figures and inferential statistics.

IV. Results

In table-1: Shows distribution of the respondents by age where in rural group highest 23 (38.33%) respondents belong to 32-43 years and in urban group highest 31 (51.66%) respondents belong to 44-55 years. The following table is given below in detail:

Table-1: Distribution of the respondents by age (n=120)

Age group	Rural Frequency (n=60) (%)	Urban Frequency (n=60) (%)
20-31 years	13 (21.67)	06 (10)
32-43 years	23 (38.33)	16 (26.67)
44-55 years	18 (30)	31 (51.66)
55 above years	06 (10)	7 (11.67)
Total	60 (100%)	60 (100%)

In figure-1 shows gender distribution of the study group where in both group majority of the cases were male. The following figure is given below in detail:

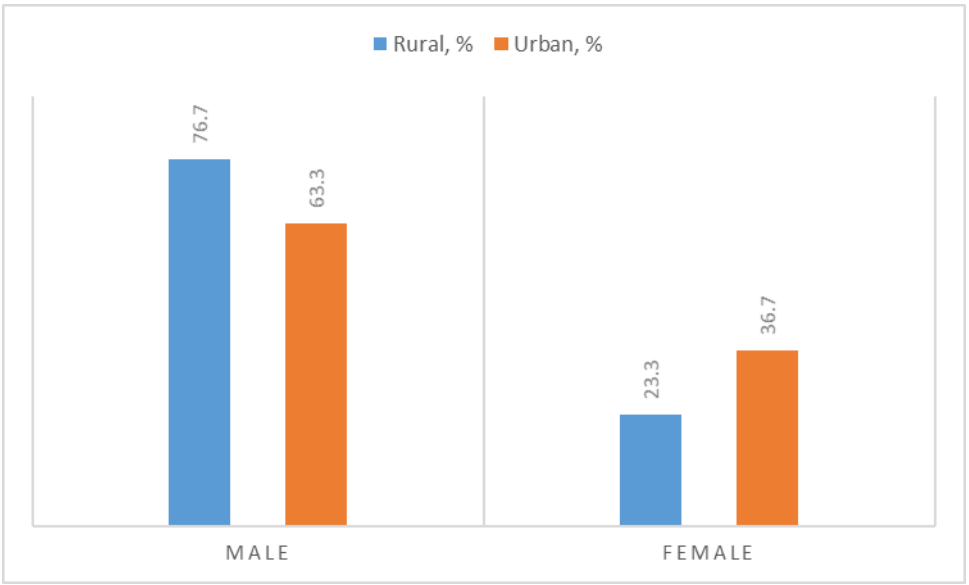


Figure-1: Gender distribution of the study group.

It was found from the table no. 2 that in rural area highest 30 (50%) respondents were HSC passed followed by primary 13 (21.7%) and in urban area highest 25 (41.25%) respondents were HSC followed by graduate 18 (30%). The bellow table is given in details.

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Table-2: Distribution of the respondents by Education (n=120)

Education	Rural Frequency (n=60) (%)	Urban Frequency (n=60) (%)
Primary	13 (21.7)	05 (8.33)
SSC	05 (8.33)	12(20)
HSC	30 (50)	25 (41.67)
Graduate	12 (20)	18 (30)
Total	60 (100%)	60 (100%)

Table-4 Explores the distribution of the respondents by knowledge related factors and association with gender of rural & urban area. The association of Covid-19 infected status between rural & urban respondents were highly significant (P= 0.00), similarly knowledge regarding first vaccine in Bangladesh also highly significant (p=0.02) between rural & urban respondents. Another findings regarding knowledge related factor regarding the lowest age range for this vaccination was moderately significant (p=0.03), finally knowledge related factor regarding the way to take the vaccine was also significantly associated (p=0.04) between the respondents of rural & urban area, beside the knowledge related factors the other variables were not significantly associated. The following table is given below in detail:

Table-4: Distribution of the respondents by knowledge related factors and association with gender (n=120)

Knowledge items	Rural		Urban		P value
	Male Frequency (n=46) (%)	Female Frequency (n=14) (%)	Male Frequency (n=38) (%)	Female (n=22) (%)	
Have you ever been infected to covid-19? • Yes • No	 22 (47.8) 24 (52.2)	 8 (57.14) 6 (42.86)	 22 (57.89) 16 (42.11)	 12 (54.54) 10 (45.46)	0.00
Any of your family member ever got infected to covid-19? • Yes • No	 25 (54.35) 21 (45.65)	 7 (50) 7 (50)	 20 (52.63) 18 (47.37)	 8 (36.36) 14 (63.64)	0.584
Do you have any Knowledge of covid-19 vaccination? • Yes • No	 38 (80.61) 8 (19.29)	 10 (71.73) 4 (28.27)	 34 (89.47) 04 (10.53)	 19 (86.36) 03 (13.64)	0.317
When did Bangladesh started corona vaccination? • January 2021 • February 2021	 33 (71.73) 13 (28.27)	 8 (57.14) 6 (42.86)	 30 (78.95) 8 (21.05)	 18 (81.81) 4 (18.19)	0.210
What is the name of first corona vaccine in Bangladesh? • COVISHIELD-Oxford • Sputnik V-Russia • Pfizer-USA • Sinovac-China	 30 (65.22) 6 (13.04) 8 (17.39) 2 (4.33)	 8 (57.14) 3 (21.42) 2 (14.28) 1 (7.16)	 30 (78.94) 8 (21.06) 00 00	 19 (83.36) 3 (16.64) 00 00	0.002
How many corona vaccines dose need to take in this country? • 1 dose • 2 doses	 5 (10.86) 41 (89.14)	 4 (28.57) 10 (71.43)	 3 (7.89) 35 (92.11)	 4 (18.18) 18 (81.81)	0.591
After the first dose of vaccination how many days it takes for the second dose? • 60 days • 45 days	 40 (86.95) 06 (3.05)	 12 (85.71) 02 (14.29)	 35 (92.10) 3 (7.90)	 22 (91.66) 02 (8.34)	0.378

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What is the lowest age range for this vaccination? • 25 years • 40 years	 20 (43.47) 26 (56.53)	 4 (28.57) 10 (71.43)	 8 (21.05) 30 (78.95)	 05 (22.72) 17 (77.28)	0.03
What is the way to take this vaccine? • Present directly to the health center • Firstly, to register on the web website • I don't know	 8 (17.39) 34 (73.91) 04 (8.70)	 04 (28.57) 08 (57.14) 02 (14.29)	 8 (21.06) 30 (78.94) 00	 4 (18.18) 18 (18.82) 00	0.041

Note: p ≤ 0.05 considered as significant value

Table-5 Find out the *distribution of each attitude and practice item and gender of* rural & urban area. The association of Covid-19 vaccine taken status between rural & urban respondents were highly significant (P= 0.00), similarly the opinion regarding so many rumors about corona vaccine also moderately significant (p=0.03) others *attitude and practice* related factors were not significantly associated. The following table is given below in detail:

Table-5: Distribution of each attitude and practice item and gender difference (n=120)

Description	Rural		Urban		P value
	Male n=46 %	Female n=14 %	Male, n=38 %	Female n=22 %	
Have you taken the vaccine? • Yes • No	 16 (34.78) 30 (65.22)	 5 (35.71) 9 (64.29)	 25(65.79) 13 (34.21)	 17 (77.27) 5 (22.73)	0.00
Is there any side effect of corona vaccination? • Yes • No	 14 (30.43) 32 (69.57)	 4 (28.57) 10 (71.43)	 8 (21.05) 30 (78.95)	 4 (18.18) 18 (81.82)	0.148
After taking the vaccine is there any side effect seen? • Fever • Body ache • Pain in inject area • Others	 09 (19.56) 15 (32.61) 20 (43.48) 02 (4.35)	 02 (14.29) 03 (21.43) 08 (57.14) 01 (7.14)	 06 (15.79) 10 (26.32) 22 (57.89) 00	 02 (9.09) 08 (36.36) 12 (54.55) 00	0.256
There are so many rumors about corona vaccine, do you believe those? • Yes • No	 12 (26.09) 34 (73.91)	 5 (35.71) 9 (64.29)	 03 (7.89) 35 (92.11)	 4 (18.18) 18 (81.82)	0.039
Are you tensed about the side effect of corona vaccine? • Yes • No	 12 (26.09) 34 (73.91)	 5 (35.71) 9 (64.29)	 05 (13.16) 33 (86.84)	 04 (18.18) 18 (81.82)	0.120
Do you think the related person should be more concern about corona vaccine? • Yes • No	 32 (69.57) 14 (30.43)	 9 (64.29) 5 (35.71)	 33 (86.84) 05 (13.16)	 18 (81.82) 04 (22.73)	0.119
Would you share the right information to your family friends and society? • Yes • No	 46 (100) 00	 14 (100) 00	 38 (100) 00	 22 (100) 00	0.163

Note: p ≤ 0.05 considered as significant value

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V. Discussion

During the study, knowledge was significantly associated with education, monthly income of a family, and previous vaccine uptake experience. However, attitudes were significantly associated with only gender and earlier vaccine administration experience. Importantly, the majority of participants showed positive attitude towards COVID-19 vaccine.

Knowledge regarding COVID-19 vaccinations negative correlation observed in terms of participants' gender. This finding is similar to studies concerning knowledge towards COVID-19 (not vaccinations) conducted in Bangladesh which reported that males had marginally higher scores in knowledge regarding COVID-19 than females. [7]

However, this finding is inconsistent to studies concerning knowledge towards COVID-19 (not vaccinations) conducted in Bangladesh which reported that males had marginally higher scores in knowledge regarding COVID-19 than females. [8]

These discrepancies of knowledge found in our study on COVID-19 vaccinations are possibly due to limited government exposures to information or publicity on COVID-19 vaccinations since the vaccine rollout started. In addition, the potential under-reporting or misinformation of data on the seriousness of incidence and mortality of COVID-19 may reduce concerns about vaccine safety or indeed make residents of Bangladesh reluctant to seek information on either COVID-19 or related vaccinations. Thus, it is essential to support community members by providing easy access to trusted, evidence-based vaccine information. [9]

According to our study, participants with a higher level of education were found to have more knowledge about COVID-19 vaccinations, which is also supported by previous research. Similar scenarios were found in other earlier studies in Bangladesh, illustrating that individuals with a higher educational background showed more knowledge regarding COVID-19. [10]

It may be the case that more educated people are more knowledgeable and concerned about their health and well-being, through access to more information sources, and become more engaged in life events that could impact them, such as COVID-19 vaccinations. [11]

People who have received any vaccine earlier were found to have more knowledge regarding COVID-19 vaccinations in this study. A recent study in China evaluating COVID-19 vaccine acceptance found that people who were previously vaccinated against influenza were more likely to accept the COVID-19 vaccine, which was also demonstrated in a study in Hong Kong.[12,3]

This tendency among people may be due to previous positive experiences from vaccination. The level of knowledge about COVID-19 vaccinations were significantly higher among people living in the urban areas, compared to rural areas. This is supported by an earlier study in Bangladesh which demonstrated significant correlation between COVID-19 knowledge and urban location.[7] However, our finding is inconsistent with a recent study which found more accurate knowledge about COVID-19 among people in rural areas in Bangladesh.[8]

In the present study, over 80% of participants had more positive attitudes towards COVID-19 vaccine. This association is in line with a previous study on attitudes towards dengue vaccination conducted in Indonesia [35] and attitudes towards COVID-19 carried out in Bangladesh. [8]

In our study, in participants assumed that the recently discovered COVID-19 vaccine (the vaccine currently being used in Bangladesh) could have some side-effect, which is similar to a study in the US.[13] A study in China found that 48% of respondents postponed vaccination before confirmation of the safety of the vaccine, which shows their doubt regarding vaccine safety.[14] Worryingly, the exceptionally rapid pace of vaccine development, the skepticism of certain groups of science and health experts might elevate doubt about COVID-19 vaccine. [15]

VI. Conclusion

The COVID-19 pandemic is still experiencing worldwide disasters and lives, but a possible ray of hope for the future can be found with the COVID-19 vaccine. The findings recommend immediate programs of health education and that the respective health authorities should provide more accurate information. In order to decrease vaccine relief enabled and promoted by disinformation in the media, policymakers should take efforts to provide appropriate understanding, favorable attitudes and views of COVID-19 immunization.

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The Impact of *Senna Siamea* (Lam) Leaves Extracts on 2, 2-Dichlorovinyldimethyl Phosphate Induced Brain Oxidative Stress in Wistar Albino Rats

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Abstract- Organophosphate compounds have been a common source of mortality in recent times. A typical example is dichlorvos [2, 2-Dichlorovinyldimethylphosphate (DDVP)]; an important agricultural pesticide. This study sought to investigate the phytochemical screening, in-vitro antioxidant property of the leaf solvents extracts and ameliorating effects of Senna siameain DDVP-induced brain oxidative stress in Wistar rats. The aqueous, methanol and ethanol extracts of the leaves were obtained and analyzed for their in-vitro antioxidant parameters. Thirty-two healthy Wistar albino rats were grouped into eight of 4rats each weighing between 140-150g. The animals were orally administered with 6.6mg/kg body weight of DDVP except groups1 and 2 followed by treatments with the three solvents extracts respectively that lasted for four weeks.

Keywords: *Senna siamea; brain toxicity; oxidative stress; antioxidant properties; γ-glutamyl transferase.*

GJMR-B Classification: *NLMC Code: WB 340*



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The Impact of *Senna Siamea* (Lam) Leaves Extracts on 2, 2-Dichlorovinyldimethyl Phosphate Induced Brain Oxidative Stress in Wistar Albino Rats

Oni, Olaiya Peter [α], Oseni, Olatunde Abass [σ] & Okoh, Olayinka Sunday [ρ]

Abstract- Organophosphate compounds have been a common source of mortality in recent times. A typical example is dichlorvos [2, 2-Dichlorovinyldimethylphosphate (DDVP)]; an important agricultural pesticide. This study sought to investigate the phytochemical screening, in-vitro antioxidant property of the leaf solvents extracts and ameliorating effects of *Senna siameain* DDVP-induced brain oxidative stress in Wistar rats. The aqueous, methanol and ethanol extracts of the leaves were obtained and analyzed for their in-vitro antioxidant parameters. Thirty-two healthy Wistar albino rats were grouped into eight of 4rats each weighing between 140-150g. The animals were orally administered with 6.6mg/kg body weight of DDVP except groups1 and 2 followed by treatments with the three solvents extracts respectively that lasted for four weeks. The in-vitro antioxidant potentials of the plant extracts and malondialdehyde (MDA); reduced glutathione (GSH) concentrations; γ-glutamyl transferase (GGT); catalase (CAT); superoxide dismutase (SOD); glutathione peroxidase (GPx); glutathione transferase (GST) and glutathione reductase (GR)activities were determined on the serum and brain homogenate. The three plant extracts were found to possess significant in-vitro antioxidant potentials while treatments of the DDVP induced brain toxicity with the extracts caused significant reduction in the raised values of MDA concentration and GGT activity with increased antioxidant enzymes activities when compared to the DDVP-induced group. The results obtained from the study thus showed that the leaves extracts contained important phytochemicals, possessed in-vitro and in-vivo antioxidant potentials responsible for protective and curative effects against DDVP-induced brain oxidative stress of the rats.

Keywords: Senna siamea; brain toxicity; oxidative stress; antioxidant properties; γ-glutamyl transferase.

I. Introduction

Exposure of human beings to poisons and some other toxic materials has been responsible for many mortality cases in our generation most of which are accidental. Reports indicate that nothing less than 200,000 were dead as a result of organophosphate compound, one of which is 2, 2- Dichlorovinyldi-methylphosphate (DDVP)[1]. The organophosphate compounds is unarguably one of the toxic and adequately chronic organophosphates that is of detrimental effect to the health of humans and animal[2]. It was said that the continuous exposure to humans and animals to DDVP has been identified as one of the leading causes of acetyl cholinesterase (AChE) inhibition especially at the presynaptic cleft, thus leading to the accumulation of acetylcholine as well as the triggering of postsynaptic neurons in animals, and ultimately to death[3]. Notably, the exposure of humans and animals have been fingered to be a key player in respiratory problems including that of discomfort in the chest, bloody or running nose, severe and sometimes dry coughing, difficulty in breathing and increased fluid in the bronchial tubes[4]. Oxidative stress arising from free radicals like reactive oxygen species (ROS) now appears to be a fundamental mechanism underlying many degenerative diseases such as diabetes, viral infection, auto-immune pathologies and probably aging. Evidence suggests that ROS can be scavenged through chemoprevention utilizing antioxidant compounds present in foods and medicinal plants[5]. Plants play a significant role in maintaining human health and improving the quality of human life. They serve humans as valuable components of food, cosmetics, dyes, and medicines. The World Health Organization estimated that 80% of the earth's inhabitants rely on traditional medicine for their primary healthcare needs, and most of this therapy involves the use of plant extracts and their active components[6]. *Senna siamea* has a long history of use as a folk-medicine and its therapeutic efficacy is well recognized. Different parts of *S. siamea* can be used for various medical purposes[7;8;9]. The fruit is used to charm away intestinal worms and to prevent convulsions in children. The heartwood is said to be a laxative, and a decoction is used against scabies[10]. *Senna siamea* also known as Siamese cassia, kassod tree, cassod tree and cassia tree is a legume in the subfamily Caesalpinioideae. It is native to South and Southeast Asia, widespread in Africa, although its exact origin is unknown11. This plant has proven to contain some important biochemical components like alkaloids, volatile essential oils, phenols and phenolic glycosides, resins, oleosins, steroids, tannins and terpenes. *Senna siamea*is a medicinal plant acknowledged to be rich in phenolics, consisting of condensed tannin and phlobatannin, Gallic acid, protocatechuic acid,

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pyrocatechol, (+)-catechin, (-) epi-gallocatechin-7-gallate and (-) epigallocatechin-5, 7-digallate[9,12]. Its antioxidative property and ameliorating effects on organophosphate toxicity has not been done. This current study however tends to investigate the effects of *Senna siamea* leaf extracts on some biochemical indices in 2, 2-dichlorovinyldimetrhyl phosphate (DDVP) induced brain toxicity using Wistar albino rats.

II. Materials and Methods

a) Collection and Extraction of the Senna siamea leaf

The fresh leaves of *Senna siamea* (Lam) Irwin & Barneby (Fabaceae) were obtained from Ifaki-Ekiti community, Ekiti State and was authenticated at Department of Plant Science, Ekiti State University, Ado-Ekiti and the plant specimen was preserved with Herbarium numbers (UHAE 2020055). The leaves were rinsed with water and then air-dried by spreading them on a clean surface at room temperature in the laboratory. The air-dried leaves were then pulverized and three major separate extractions were carried out with two hundred grams portions each of the dried powdered leaves soaked in 500mL each of water, ethanol and methanol as solvents to obtain three different extracts. The extracts were then concentrated by increased surface area evaporation to obtain dried extracts for analyses.

b) Phytochemical screening and in-vitro anti-oxidant parameters determination of the leaf's extracts

The qualitative phytochemical screening [flavonoids, saponin, phlobatannins, terpenoids, Salkowski test for cardiac glycosides (steroidal ring or terpenoids), Keller-Killani test for cardiac glycosides (deoxysugar), Lieberman's test for steroidal nucleus and test for tannins] of aqueous, methanol and ethanol extracts of the leaves were carried out according to the methods of[13,14]to identify the active constituents while the in-vitro antioxidants properties were determined by the following methods:

a. Hydrogen Peroxide Scavenging Effects: The ability of the leaf extracts to scavenge hydrogen peroxide was assessed by the method of Ruch *et al* [15].
b. ABTS Scavenging Effects: The antioxidant effect of the leaf extracts was studied using ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical cationde colourisation assay according to the method of Shirwaikar *et al* [16].
c. Measurement of Nitric Oxide Scavenging Activity: The extent of inhibition of nitric oxide radical generation *in vitro* was followed as per the method reported by Green *et al*[17].
d. DPPH spectrophotometric assay: The free radical scavenging activities of the samples by DPPH method were determined according to the method reported by Brand-Williams *et al*[18].
e. Measurement of Superoxide Scavenging Activity: The superoxide scavenging ability of the extracts was assessed by the method of Winter bourn *et al*[19].
f. Estimation of Total Phenols: The total phenolic content was determined according to a well-cited protocol[20].
g. Estimation of Flavonoids: The total flavonoid contents in the samples were determined following the method reported by Zhishen *et al*[21].

c) Animal management

Thirty-two (32) healthy albino Wistar rats were obtained and housed in the animal house of the College of Medicine, Ekiti State University, Ado- Ekiti, Nigeria. The animals were acclimatized for two weeks before administration of DDVP. The acclimatization was done under standard environmental conditions of good lighting, moderate temperature and adequate ventilation. They were also fed on standard rat feed containing adequate proteins, carbohydrate, fats, vitamins, minerals back up with clean and adequate water. The animals were handled under standard laboratory protocols as stipulated by the Institutional Animal Care and Use Committee[22].

d) Experimental design

The animals were divided into six groups according to their weights with Groups 2 having 3 subgroups, one for each of the three extracts. Each group had four animals. The animals were orally administered with 0.5mL of 6.6mg/kg body weight of 500 folds dilution of DDVP solution for two weeks except for Groups 1 and 2 followed by treatments with 0.5mL of 3.3mg/kg body weight of each extract of the plant for another two weeks of the four weeks study.

Group 1 Normal Control

Group 2 Extract control (Each subgroup animal was given 0.5mL of 3.3mg/kg body weight of 0.5g/100mL of each solvent (aqueous, methanolic and ethanolic) extract of the plant)

Group 3 DDVP control (animals were administered orally with 0.5mL of 6.6mg/kg body weight of 500 folds dilution of DDVP solution to induce brain toxicity)

Group 4 DDVP induced animals + 0.5mL of 3.3mg/kg body weight of 0.5g/100mL aqueous extract of the plant.

Group 5 DDVP induced animals + 0.5mL of 3.3mg/kg body weight of 0.5g/100mL methanolic extract of the plant.

Group 6 DDVP induced animals + 0.5mL of 3.3mg/kg body weight of 0.5g/100mL ethanolic extract of the plant.

All animals in the groups were also given rats feed and drinking water *ad libitum.*

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e) Preparation of serum and brain homogenate

At the end of the experiment, the rats were chloroform anesthetized and quickly dissected with their blood samples and brain removed. 10% of the brain homogenate was prepared in 6.7nM potassium phosphate buffer (pH 7.4) using the Top driven electric homogenizer. The homogenate was centrifuged at 3,000rpm for 10 minutes at 4^0C to obtain a clear supernatant while serum sample was prepared from the whole blood collected from the heart into the plain sample bottle and centrifuged at 3,000 rpm after coagulation. The individual serum and brain homogenate were used for measurement of the studied biochemical parameters. The lipid peroxidation was done by measuring the TBARS in accordance with the modified method of Utley et al[23]; GGT activity was determined using standard Sigma-Aldrich[24] kit from USA while the antioxidants enzymes activities [Catalase (CAT), superoxide dismutase (SOD), Glutathione-S-transferase (GST), Glutathione reductase (GR) and Glutathione peroxidase (GPx)]; reduced glutathione (GSH) were determined by the methods described by Chance and Maehly[25] as calculated by Von Euler and Josephson[26]; Misra and Fridovich[27]; Habig*et al*[28]; Carlberg and Mannervik[29]; Mohandas et al[30] and Jollow et al[31] respectively.

f) Statistical analyses

The results obtained were evaluated using the statistical test of Means triplicates results of four animals per group.

III. Results

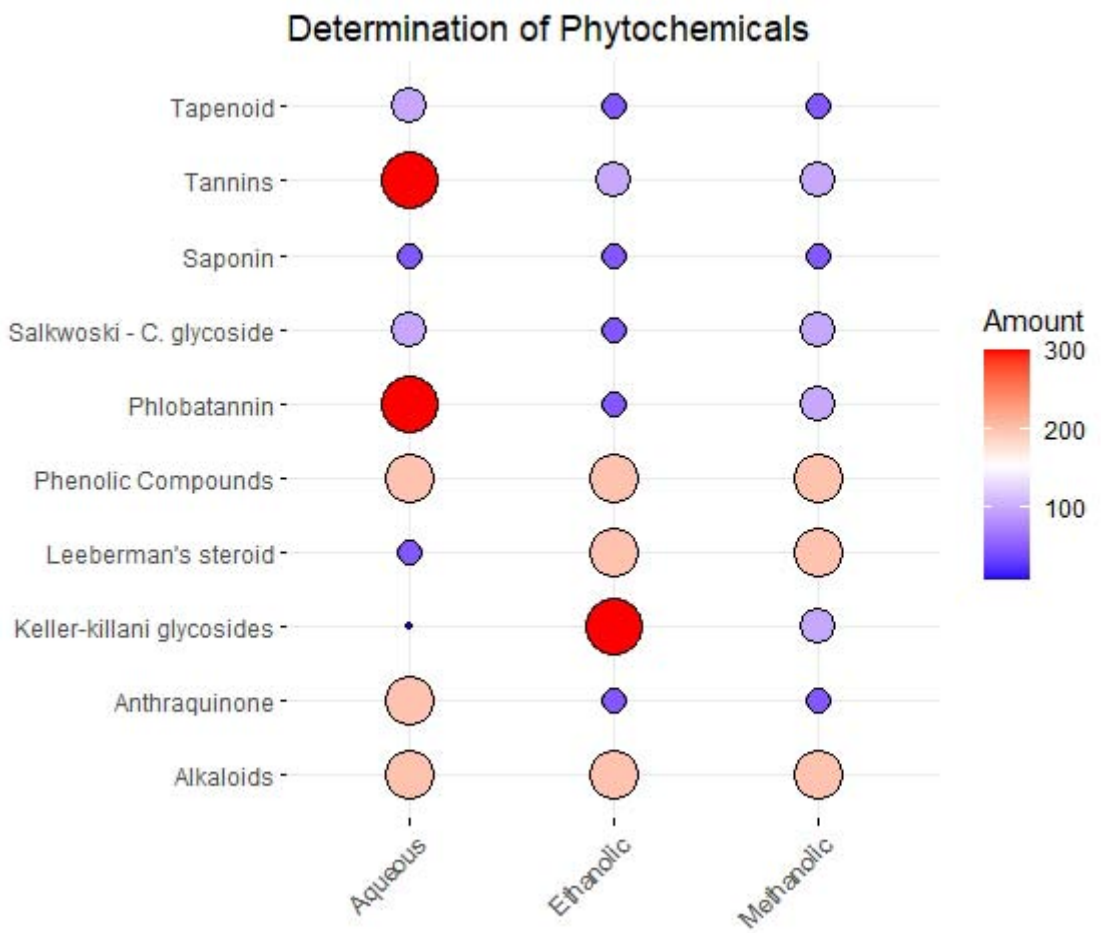


Figure 1: Phytochemical screening of the composition of aqueous, methanol and ethanol extracts of *Senna siamea* leaf. Less than 100 means trace of the phytochemicals, 100 means "+"; 200 means "++"; while 300 means "+++".

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Figure 2: In-vitro anti-oxidants determination of the aqueous, methanol and ethanol extracts of *Senna siamea* leaves at 0.25, 0.50, 0.75 and 1.00mg/mL concentrations of the extracts.

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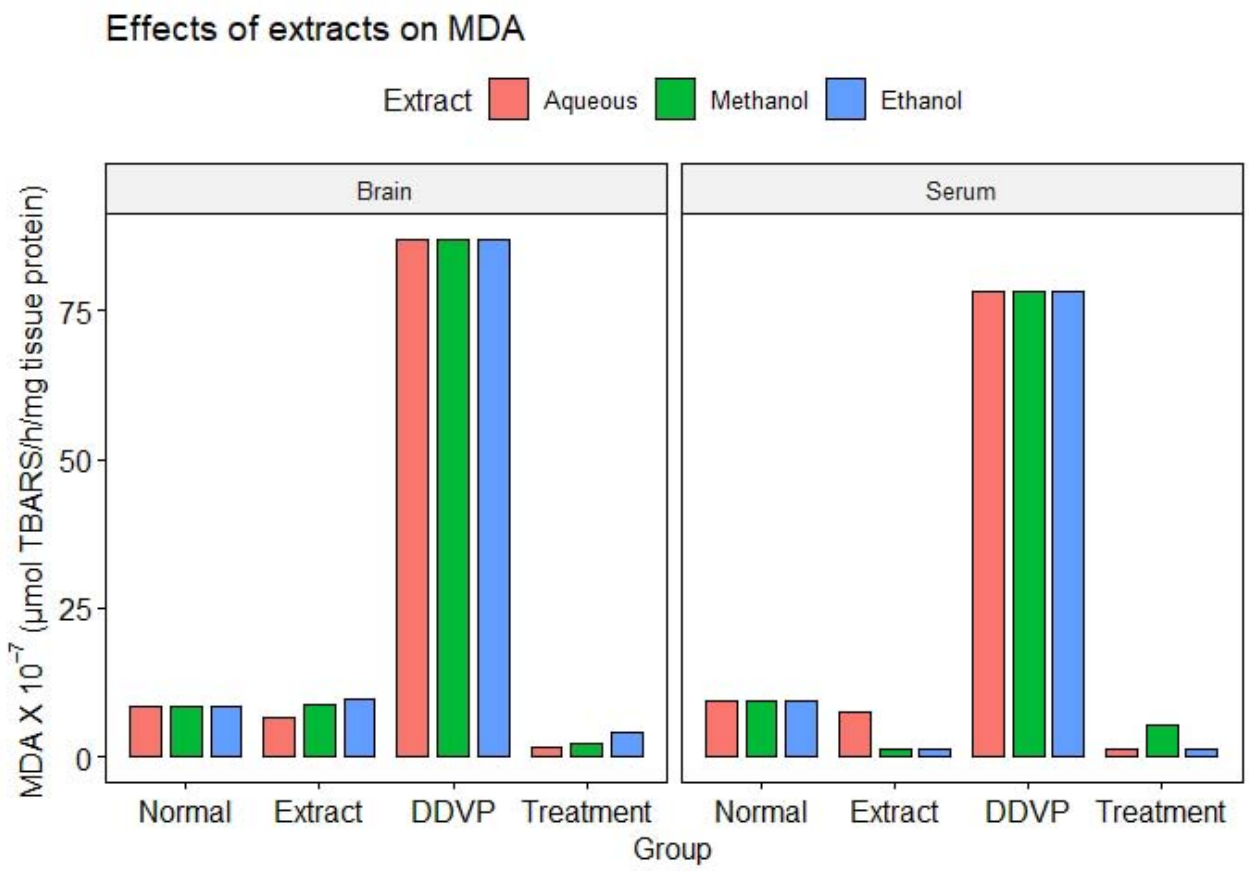


Figure 3: Effect aqueous, methanol and ethanol extracts of *Senna siamea* leaves on MDA (µmolTBARS/h/mg tissue protein) in 2, 2-Dichlorovinyldimethylphosphate (DDVP)-induced toxicity in Wistar albino rats. Normal Group animals were given water and feed only, Extract Group animals were given the extracts, while the DDVP Group animals were induced with DDVP and the Treatment Group animals were treated with the *Senna siamea* leaves extracts after being induced with DDVP.

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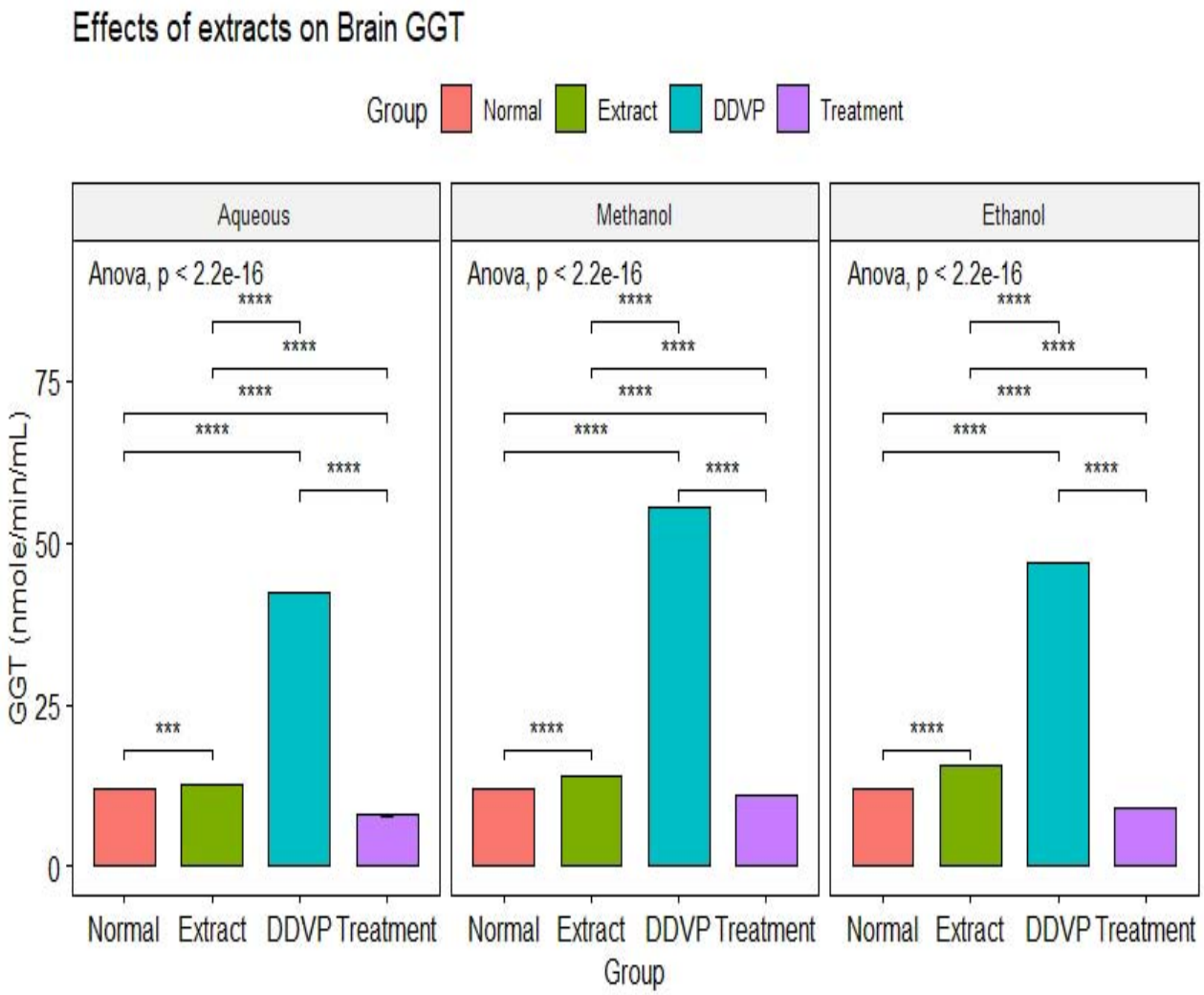


Figure 4: Effect of *Senna siamea* extracts on Brain γ-glutamyl transferase -GGT (nmole/min/mL) in 2,2-Dichlorovinyldimethylphosphate (DDVP) induced toxicity in Wistar albino rats. $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001<$ (****). Normal Group rats received feeds and water only; Extract Group rats received one of aqueous, methanol or ethanol extract of *Senna siamea* in addition to feeds and water; DDVP Group rats were induced with DDVP while the Treatment Group rats received one of aqueous, methanol or ethanol extract of *Senna siamea* after exposure to DDVP.

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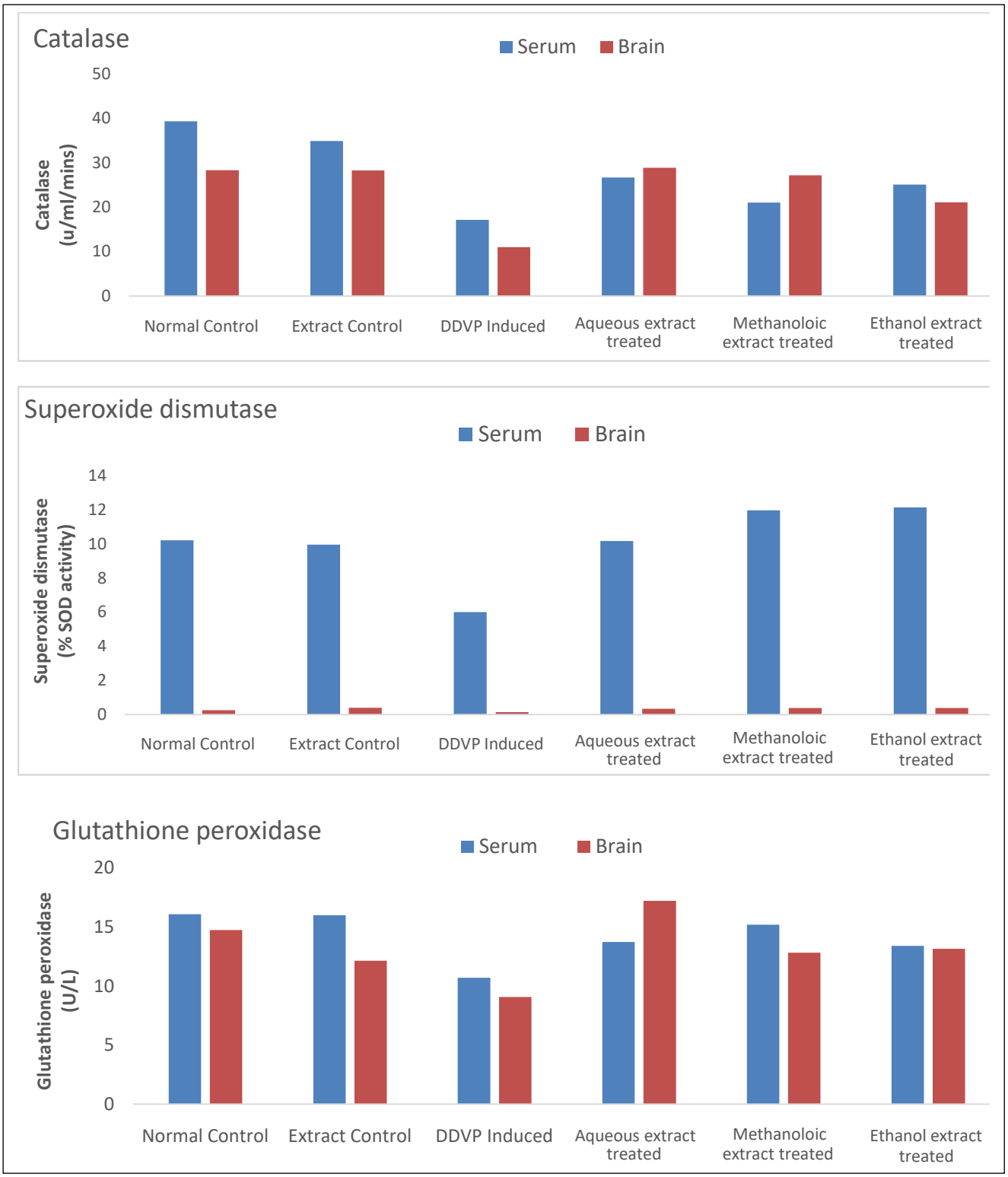


Figure 5a: The effect of DDVP – induced toxicity on Catalase, Superoxide Dismutase, Glutathione Peroxidase and Glutathione Transferase in Wistar albino rats.

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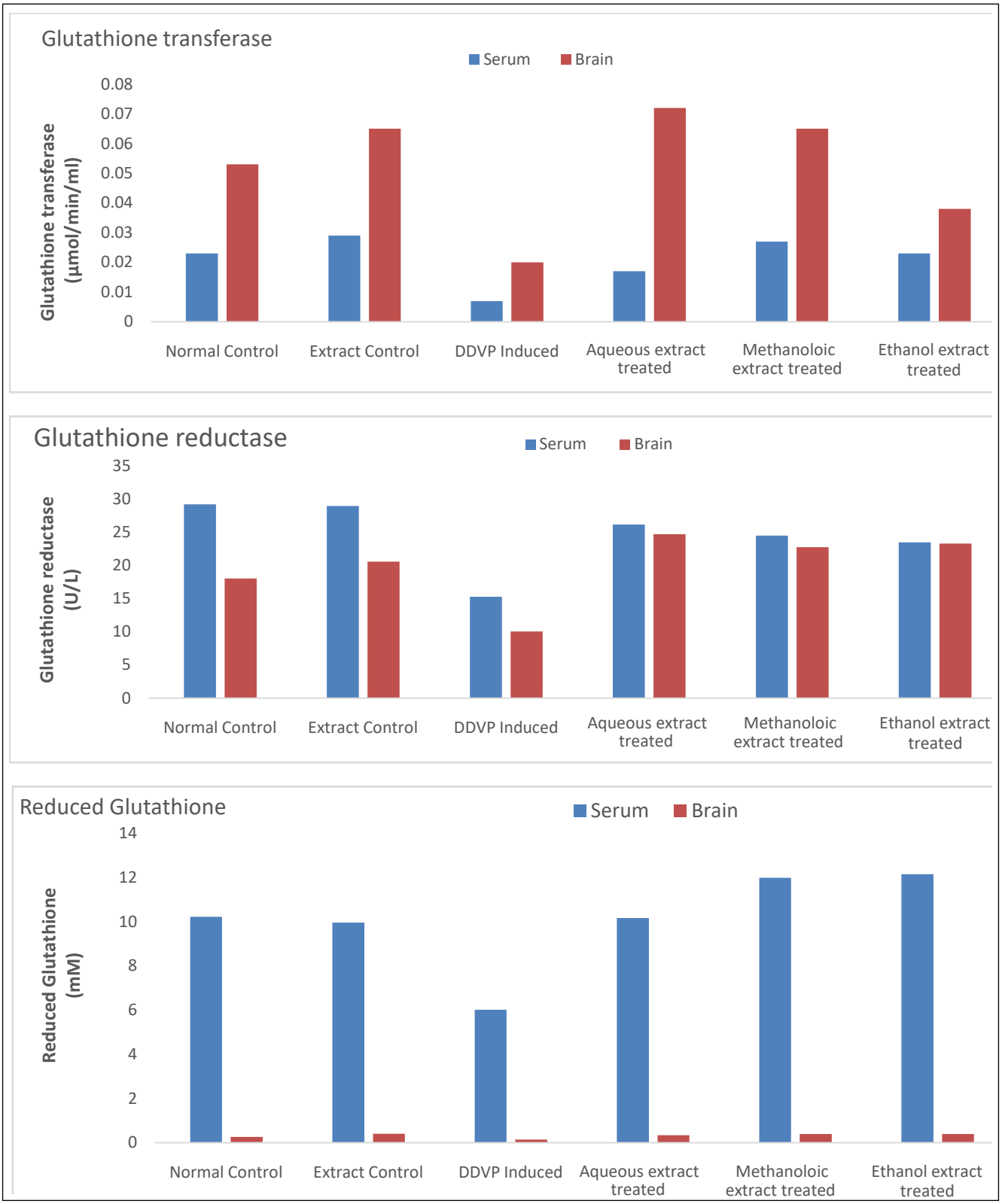


Figure 5b: The effect of DDVP – induced toxicity on Glutathione reductase and Reduced glutathione in Wistar albino rats.

IV. Discussion

The Phytochemical assessment of the three solvents extracts of the leaves of *Senna siamea* contained some plant chemicals of interest and important medicinal potentials as shown in Figure 1.0. This important plant phytochemicals are known to support bioactive activities in medicinal plants and may

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therefore be responsible for the medicinal properties of these leaves extracts which was similar to many other reported studies by[32] in protective effects of the ethanolic extract of *Alstonia boonei* stem bark and[33] in in-vitro compositional investigations of antioxidants, phytochemicals, nutritional and minerals in the fruit of *Kigelia africana* (Lam.) Benth. Figure 2.0 related the in-vitro antioxidant properties of the plants extracts studied within 0.25 and 1.0mg/mL concentrations range for both extracts. The % H_20_2, %ABTS, %NO, %DPPH and %SO scavenging activities which occurred in a concentration dependent fashion similar to what was obtained for total phenol and total flavonoids concentrations which are the bioactive compounds responsible for the antioxidant potentials of the *Senna siamea* various solvents extracts. The three extracts of the leaves showed appreciable scavenging potentials for nitric oxide, the nitric oxide scavenging property of any plant extract has been said to help in the arrest of various chains of reactions initiated by excess generation of NO that are detrimental to the wellbeing of the body. The results obtained in this study however corroborated the earlier obtained for the nitric oxide scavenging activity of *Ceropsdecandra*[34]. The three extracts of the leaves showed appreciable scavenging potentials for DPPH radicals in a concentration dependent manner. indicating that the higher the concentration used, the higher the scavenging activity with the highest scavenging activity found in methanol extract. The ability of the plant to freely scavenge DPPH radicals may be due to the presence of flavonoids[35]. The scavenging of DPPH radical by antioxidants agents is due to the reaction between antioxidant molecules and radical progress which results in the scavenging of the radicals by hydrogen donation. It is visually noticeable as a change in colour from purple to yellow. Hence, DPPH is usually used as a substance to evaluate the antioxidant potential of medicinal plants[36]. The Ethanolic extract of *Senna siamea* displayed a more superior superoxide scavenging activity at the 1mg/mL concentration which is far greater than the results for both aqueous and methanolic extracts at the same concentration. The super oxide scavenging activity of *Senna siamea* can be seen in its ability to scavenge super oxide radical ions to form stable radicals and by such can help in the termination of radical chain reaction[37].In the hydrogen peroxide scavenging activity, the ethanolic extracts possess the highest activity than those aqueous and methanol. However, Shalu*etal*[38] reported a similar observation for the plant in their study. Total phenols and total flavonoids were also observed to be considerably present in all the plant extracts. The total phenol aqueous extract had the highest value followed closely by the ethanolic and methanolic extracts concentrations. These results obtained for *Senna siamea* showed resemblance to the work of Jyoti*etal*[39] on *Acacia nilotica.* Phenols are said to contribute to the quality and nutritional value in terms of modifying aroma, color, taste and flavor[39]. Phenolic compounds could be a major determinant of antioxidant potentials of food plants and could therefore be a natural source of antioxidants[40]. In this study, the results obtained for total flavonoids showed that ethanolic extract contained the highest level of flavonoids than aqueous and methanolic extracts. Flavonoids are part of the secondary metabolites present in plants as part of its arsenal and has been reported by Choudhary*etal*[40] that flavonoids show some antioxidant activity and that it has considerable effects on both human's health and nutrition. He described its mechanisms as the one with either scavenging or chelating process. They are said to possess hydroxyl groups which prompted their radical scavenging effects in the plant. Figure 3.0 showed the effects of the leaf extracts on the level of lipid peroxidation both in the serum and brain of the studied animals which revealed that the DDVP induced control group 3 caused almost tenfold elevation in the malondialdehyde concentration of both serum and brain tissue. The treatment of the rats with the three solvents extracts showed that both extracts produced ten-fold reduction effects in the concentration of malondialdehyde in both serum and brain tissues when compared with the DDVP-induced group. It has been reported that enhanced lipid peroxidation leads to tissue damage and failure of antioxidant defense mechanism to prevent formation of excessive free radicals[41]. It is clearly evidenced in this study that treatments of the induced animals with the aqueous, methanolic and ethanolic extracts of the *Senna siamea* leaf respectively caused a substantive decrease in the level of lipid peroxidation of both serum and brain tissues. This result however corroborated the observation of Ojo *et al*[32] who postulated that DDVP induction would lead to an increased MDA value and the treatment with antioxidant containing extracts would lead to the reduction of the MDA value. The induction of DDVP in group 3from Figure4.0 showed a significantly increase in the value of GGT which thus implies that the exposure caused a damage to the brain including increased permeability, possible neurosis in the brain. This result also showed that the treatment with the various solvents extracts caused a significant decrease in the level of the GGT indicating that the extracts of *Senna siamea* caused reversal to the detrimental DDVP induction in the animals. This is in line with the results of Ojo *et. al*[32] who also postulated that DDVP induction will lead to an increased GGT value and the treatment with a good antioxidant plant like *Senna siamea* will reverse back the damage caused by the exposure to DDVP; it should also be noted in this study that all the solvents extracts of the plant produced tremendous attenuating effect of the brain toxicity. Figures 5a and 25b presented the results of some antioxidant enzymes of (catalase, superoxide dismutase, glutathione peroxidase,

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glutathione transferase, glutathione reductase) and reduced glutathione. Oxidative stress plays a major role in the pathogenic of many disorders including aging, cancer, diabetes, Alzheimer's, strokes, viral infections (that cause airway epithelial inflammation), neurodegenerative processes (including cell death, motor neuron diseases and axonal injury) and infraction, and brain edema. Antioxidant enzyme plays an important role in protecting oxidative injury to the body. One of the therapeutic approaches by which these disorders can be prevented is to increase the levels of these antioxidant enzymes[42]. The catalase activity was significantly increased at ($p<0.05$)in the aqueous, methanolic and ethanolic treatments groups towards the control and extract treated groups when compared with the DDVP induced group. Other researchers have also reported an upward trend in catalase level using various plants extract treatments in other complications as we observed in this study[43,44,45,46]. Catalase are heme-containing enzymes that convert hydrogen peroxide (H_2O_2) to water and O_2, and they are largely localized in subcellular organelles such as peroxisomes[47]. The %SOD activity observed in this study showed a significant ($p<0.05$) increase in the various treatment's groups after a gross reduction by the DDVP induced group when compared with the normal control and extracts treated groups. Similar observations were also reported in earlier studies of (Oseni *et al*[43]; Uroko *et al*[44]; Onoja *et al*[45] and Sani *et al*[46]). SOD is the antioxidant enzyme that catalysed the dismutation of the highlyreactive superoxide anion to O_2 and to the less reactive species H_2O_2; the peroxide can then be destroyed by Catalase or glutathione peroxidase reactions as reported by[48,49,50]. Glutathione peroxidase, glutathione transferase and glutathione reductase in addition with SOD are antioxidants enzymes that work in synergy to protect the organism from reactive oxygen species (ROS). These enzymes were observed to be significantly ($p<0.05$) increased in all the treatment groups when compared with the induced group to reverse the effects of induction towards the normal control group. Our observation is in consonance with what was reported by51,52in their various studies on amelioration of thioacetamide-induced oxidative stress and hepatic damage in albino rats by Solanum trilobatum and antioxidant effect of grapevine leaf extract on the oxidative stress induced by a high-fat diet in rats respectively. Reduced glutathione (GSH) is another compound that play a vital role as an antioxidant. *Senna siamea* solvents extracts were found to significantly ($p<0.05$) reverse the reduced GSH in DDVP induced rats to the normal control as observed in this study. Reduced glutathione is found in high concentrations in cellular systems and plays a major role in detoxication of various electrophilic compounds, deficiency of which puts the cell at risk for oxidative damage. Previous works have also shown that medicinal plants extracts have abilities to enhance glutathione concentration to reverse the effects of oxidative stress[51,53].

V. Conclusion

This study has reasonably showed that the oral exposure of the rats to DDVP (Dichloros) caused the brain oxidative stress in the Wistar albino rats as indicated by the increased level of lipid peroxidation, increased GGT activity, reduced antioxidant potentials both in the serum and brain while the aqueous, methanolic and ethanolic extracts of *Senna siamea* showed a significant and protective effects against the action of DDVP induced oxidative stress in the rats.

The plant studied here will in no doubt do well as a neurotoxicity protective agents and further researches needed to be carried out to explore it for raw materials needed for the treatment of neurodegenerative diseases like Alzheimer's diseases and other complications.

Ethics approval and consent to participate: All necessary National and International ethical considerations were fully followed in handling the animals.

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Overuse of Steroid Drugs Methylprednisolone and Dexamethasone (Oral) Causes a Diabetic Patient to Become Infected with the Black Fungus in the Corona Virus

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Abstract- *Background:* Overuse of both methylprednisolone and dexamethasone drug on a corona patient can result in serious side effects and new infections may appear during their use. Infection with any pathogen, including viral, bacterial, fungal, protozoan, or helminthic infections at any site of the body, may be associated with the use of methylprednisolone or dexamethasolone in combination with other immunosuppressive agents that increase cellular immunity, humoral immunity, or Suppress neutrophils. The function they affect.

These infections can be mild, but can be serious and sometimes fatal. With increasing doses of methylprednisolone and dexamethasone, the rate of occurrence of infectious complications increases. When methylprednisolone and dexamethasone are used, there may be reduced resistance and inability to localize the infection.

Keywords: *methylprednisolone, dexamethasone, side effect, drug interaction, diabetes patients, black fungus, eye diseases etc.*

GJMR-B Classification: *NLMC Code: QV 745*

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Overuse of Steroid Drugs Methylprednisolone and Dexamethasone (Oral) Causes a Diabetic Patient to Become Infected with the Black Fungus in the Corona Virus

Ashwin Singh Chouhan [α], Bharat Parihar [σ], Bharti Rathod [ρ] & Ramprasad Prajapat [ω]

Abstract- Background: Overuse of both methylprednisolone and dexamethasone drug on a corona patient can result in serious side effects and new infections may appear during their use. Infection with any pathogen, including viral, bacterial, fungal, protozoan, or helminthic infections at any site of the body, may be associated with the use of methylprednisolone or dexamethasolone in combination with other immunosuppressive agents that increase cellular immunity, humoral immunity, or Suppress neutrophils. The function they affect.

These infections can be mild, but can be serious and sometimes fatal. With increasing doses of methylprednisolone and dexamethasone, the rate of occurrence of infectious complications increases. When methylprednisolone and dexamethasone are used, there may be reduced resistance and inability to localize the infection.

Prolonged use of methylprednisolone and dexamethasone may produce posterior subcapsular glaucoma, glaucoma with potential damage to the optic nerves, and may accelerate the establishment of secondary ocular infections caused by fungi or viruses.

It has also been observed that more methylprednisolone and dexamethasone drug increases the level of glucose in the body, leading to normal corona patients who do not have any disease and diabetes after recovering from excessive consumption of methylprednisolone and dexamethasone drug.

Materials and Methods: A cross sectional study was conducted among 50 COVID doctors from the department's outpatient pool of COVID patients, distributing questionnaires to all subjects of different age groups. The questionnaire included information related to the name, age, gender and various factors that affect the doctor's choice of methylprednisolone and dexamethasone.

Result and Discussion: A total of 50 doctors and some medical stores from across India were included in the survey. Doctors prescribed more methylprednisolone and dexamethasone medicine than steroid medicines to corona patients. In our research, most side effects were observed for corona patients taking methylprednisolone and dexamethasone drug.

Conclusion: This research had shown that overdose of methylprednisolone and dexamethasone drug take diabetes patient he has serious eye effect and cause black fungus.

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Keywords: methylprednisolone, dexamethasone, side effect, drug interaction, diabetes patients, black fungus, eye diseases etc.

I. Background

a) Introduction

Methylprednisolone (Depo-Medrol, Medrol, Solu-Medrol) is a synthetic glucocorticoid, primarily prescribed for its anti-inflammatory and immunosuppressive effects.[1],[2],[3] It is either used at low doses for chronic illnesses or used concomitantly at high doses during acute flares. Methylprednisolone and its derivatives can be administered orally or parenterally.[4]

Regardless of route of administration, methylprednisolone integrates systemically as exhibited by its effectiveness to quickly reduce inflammation during acute flares.[5] It is associated with many adverse reactions that require tapering off the drug as soon as the disease is under control.[6] Serious side effects include iatrogenic Cushing's syndrome, hypertension, osteoporosis, diabetes, infection, and skin atrophy.[6]

Chemically, methylprednisolone is a synthetic pregnane steroid hormone derived from hydrocortisone and prednisolone. It belongs to a class of synthetic glucocorticoids and more generally, corticosteroids. It acts as a mineralocorticoid and glucocorticoid receptor agonist. In comparison to other exogenous glucocorticoids, methylprednisolone has a higher affinity to glucocorticoid receptors than to mineralocorticoid receptors.

Glucocorticoid's name was derived after the discovery of their involvement in regulating carbohydrate metabolism.[6] The cellular functions of glucocorticoids, such as methylprednisolone, are now understood to regulate homeostasis, metabolism, development, cognition, and inflammation.[6] They play a critical role in adapting and responding to environmental, physical and emotional stress.[6]

Methylprednisolone was first synthesized and manufactured by The Upjohn Company (now Pfizer) and FDA approved in the United States on October 2, 1957.[7] In 2018, it was the 153rd most commonly

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prescribed medication in the United States, with more than 4 million prescriptions.[8][9] Methylprednisolone has been a prescribed therapy amidst the COVID-19 pandemic, but there is no evidence it is either safe or effective for this purpose.[10],[11]

Dexamethasone is a glucocorticoid medication[12] used to treat rheumatic problems, a number of skin diseases, severe allergies, asthma, chronic obstructive lung disease, croup, brain swelling, eye pain following eye surgery, and along with antibiotics in tuberculosis.[12]

In adrenocortical insufficiency, it may be used in combination with a mineralocorticoid medication such as fludrocortisone.[12] In preterm labor, it may be used to improve outcomes in the baby.[12] It may be given by mouth, as an injection into a muscle, as an injection into a vein, as a topical cream or ointment for the skin or as a topical ophthalmic solution to the eye.[12] The effects of dexamethasone are frequently seen within a day and last for about three days.[12]

The long-term use of dexamethasone may result in thrush, bone loss, cataracts, easy bruising, or muscle weakness.[12] It is in pregnancy category C in the United States, meaning that it should only be used when the benefits are predicted to be greater than the risks.[13] In Australia, the oral use is category A, meaning it has been frequently used in pregnancy and not been found to cause problems to the baby.[14] It should not be taken when breastfeeding.[12]

Dexamethasone has anti-inflammatory and immunosuppressant effects. [12]

Dexamethasone was first synthesized in 1957 by Philip Showalter Hench and was approved for medical use in 1961.[15],[16],[17] It is on the World Health Organization's List of Essential Medicines.[18] In 2017, it was the 321st most commonly prescribed medication in the United States, with more than one million prescriptions.[19]

Aim: To study the factors that influence doctor's choice of methylprednisolone and dexamethasone and to understand the most preferred options in selection with respect to the methylprednisolone and dexamethasone.

II. Materials and Methods

A cross sectional study was conducted among 50 covid doctor from the outpatient pool of Department of covid patients were briefed about the study and informed consent was obtained from them and ethical committee approval was obtained from the University. Questionnaires were distributed to all subjects of various age groups. The questionnaire included information related to the covid patient's name, age, gender and various factors that influence a doctor's choice of methylprednisolone and dexamethasone.

a) Description

MEDROL Tablets contain methylprednisolone which is a glucocorticoid. Glucocorticoids are adrenocortical steroids, both naturally occurring and synthetic, which are readily absorbed from the gastrointestinal tract. Methylprednisolone occurs as a white to practically white, odorless, crystalline powder. It is sparingly soluble in alcohol, in dioxane, and in methanol, slightly soluble in acetone, and in chloroform, and very slightly soluble in ether. It is practically insoluble in water.

The chemical name for methylprednisolone is pregna - 1, 4 - diene - 3, 20-dione, 11, 17, 21-trihydroxy-6-methyl-, (6α, and 11β)-and the molecular weight is 374.48. The structural for-mula is represented below:

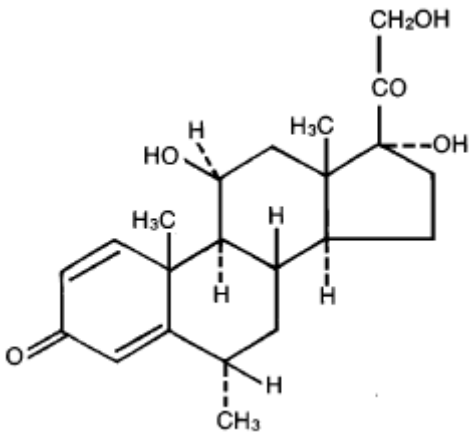


Figure No. 1: Chemical Structure of Methylprednisolone

Each MEDROL (methylprednisolone) Tablet for oral administration contains 2 mg, 4 mg, 8 mg, 16 mg or 32 mg of methylprednisolone.

DEXAMETHASONE, a synthetic adreno-cortical steroid, is a white to practically white, odorless, crystalline powder. It is stable in air. It is practically insoluble in water. The molecular formula is $C_{22}H_{29}FO_5$.

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The molecular weight is 392.47. It is designated chemically as 9-fluoro-11β, 17, 21-trihydroxy-16α-methylpregna-1, 4-diene, 3, 20-dione and the structural formula is:

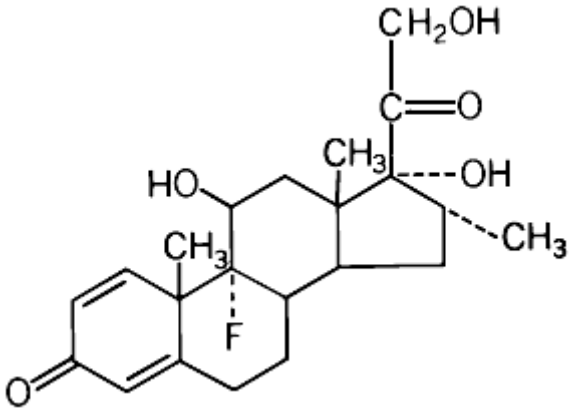


Figure No. 2: Chemical Structure of Dexamethasone.

Dexamethasone provides relief for inflamed areas of the body. It is used to treat a number of different conditions, such as inflammation (swelling), severe allergies, adrenal problems, arthritis, asthma, blood or bone marrow problems, kidney problems, skin conditions, and flare-ups of multiple sclerosis. Dexamethasone is a corticosteroid (cortisone-like medicine or steroid). It works on the immune system to help relieve swelling, redness, itching, and allergic reactions.

b) Side Effects of Methylprednisolone

✓ Fluid and Electrolyte Disturbances

Sodium retention, Congestive heart failure in susceptible patients, Hypertension, Fluid retention, Potassium loss, Hypokalemic alkalosis etc.

✓ Musculoskeletal

Muscle weakness, Loss of muscle mass, Steroid myopathy, Osteoporosis, Tendon rupture, particularly of the Achilles tendon, Vertebral compression fractures, Aseptic necrosis of femoral and humeral heads, Pathologic fracture of long bones etc.

✓ Gastrointestinal

Peptic ulcer with possible perforation and hemorrhage, Pancreatitis, Abdominal distention, Ulcerative esophagitis, Increases in alanine transaminase (ALT, SGPT), aspartate transaminase (AST, SGOT), and alkaline phosphatase have been observed following corticosteroid treatment. These changes are usually small, not associated with any clinical syndrome and are reversible upon discontinuation etc.

✓ Dermatologic

Impaired wound healing Petechiae and ecchymoses, May suppress reactions to skin tests, Thin fragile skin, Facial erythema, Increased sweating etc.

✓ Neurological

Increased intracranial pressure with papilledema (pseudo-tumor cerebri) usually after treatment, Convulsions, Vertigo, Headache etc.

✓ Endocrine

Development of Cushingoid state, Suppression of growth in children, Secondary adrenocortical and pituitary unresponsiveness, particularly in times of stress, as in, trauma, surgery or illness, Menstrual irregularities, Decreased carbohydrate tolerance.

✓ Manifestations of latent diabetes mellitus Increased requirements of insulin or oral hypoglycemic agents in diabetics

✓ Ophthalmic

Posterior subcapsular cataracts, Increased intraocular pressure, Glaucoma, Exophthalmos.

✓ Metabolic

Negative nitrogen balance due to protein catabolism

The following additional reactions have been reported following oral as well as parenteral therapy: Urticaria and other allergic, anaphylactic or hypersensitivity reactions.[22]

c) Side Effects of Dexamethasone

The following side effects have been reported with dexamethasone or other corticosteroids:

i. *Allergic Reactions*

Anaphylactoid reaction, anaphylaxis, angioedema.

ii. *Cardiovascular*

Bracardia, cardiac arrest, cardiac arrhythmias, cardiac enlargement, circulatory collapse, congestive heart failure, fat embolism, hypertension, hypertrophic, cardiomyopathy in premature infants, myocardial rupture following recent myocardial infarction, edema, pulmonary edema, syncope, tachycardia, thromboembolism, thrombophlebitis, vasculitis etc.

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iii. *Dermatologic*

Acne, allergic dermatitis, dry scaly skin, ecchymoses and petechiae, erythema, impaired wound healing, increased sweating, rash, striae, suppression of reactions to skin tests, thin fragile skin, thinning scalp hair, urticaria etc.

iv. *Endocrine*

Decrease carbohydrate and glucose tolerance, development of cushingoid state, hyperglycemia, glycosuria, hirsutism, hypertrichosis, increased requirements for insulin or oral hypoglycemic agents in diabetes, manifestations of latent diabetes mellitus, menstrual irregularities, secondary adrenocortical and pituitary unresponsiveness (particularly in times of stress, as in trauma, surgery, or illness), suppression of growth in pediatric patients.

v. *Fluid and Electrolyte Disturbances*

Congestive heart failure in susceptible patient's fluid retention, hypokalemic alkalosis, potassium loss, sodium retention etc.

vi. *Gastrointestinal*

Abdominal distention, elevation in serum liver enzyme levels (usually reversible upon discontinuation), hepatomegaly, increased appetite, nausea, pancreatitis, peptic ulcer with possible perforation and hemorrhage, perforation of the small and large intestine (particularly in patients with inflammatory bowel disease), ulcerative esophagitis.

vii. *Metabolic*

Negative nitrogen balance due to protein catabolism.

viii. *Musculoskeletal*

Aseptic necrosis of femoral and humeral heads, loss of muscle mass, muscle weakness, osteoporosis, and pathologic fracture of long bones, steroid myopathy, tenson rupture, and vertebral compression fractures.

ix. *Neurological/Psychiatric*

Convulsions, depression, emotional instability, euphoria, headache, increased intracranial pressure with papilledema (pseudotumor cerebri) usually following discontinuation of treatment, insomnia, mood swings, neuritis, neuropathy, paresthesia, personality changes, psychic disorders, vertigo etc.

x. *Ophthalmic*

Exophthalmos, glaucoma, increased intraocular pressure, posterior subcapsular cataracts.[23]

d) Drugs Interaction

DECADRON may interact with aminoglutethimide, potassium-depleting agents (e.g., amphotericin B, diuretics), macrolide antibiotics, anticholinesterases, oral anticoagulants, antidiabetics, antitubercular drugs, cholestyramine, cyclosporine, dexamethasone suppression tests (DST), digitalis glycosides, ephedrine, estrogens and oral contraceptives, barbiturates, phenytoin, carbamazepine, rifampin, ketoconazole, aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs), phenytoin, skin tests, thalidomide, and live or inactivated vaccines. Tell your doctor all medications and supplements you use and all vaccines you recently received. Decadron should be used during pregnancy or during breastfeeding only if the potential benefit justifies the potential risk to the fetus or infant. Infants may suffer adrenal suppression if their mothers use this drug during pregnancy. In special instances (for example, leukemia and nephrotic syndrome), Decadron has been used in pediatric patients. Such use should be done in most patients in conjunction with a pediatric specialist.

MEDROL may interact with aspirin (taken on a daily basis or at high doses), diuretics (water pills), blood thinner, cyclosporine, insulin or oral diabetes medications, ketoconazole, rifampin, seizure medications, or "live" vaccines. Tell your doctor all medications and supplements you use and all vaccines you recently received.[24]

Corticosteroids may mask some signs of infection, and new infections may appear during their use. Infections with any pathogen including viral, bacterial, fungal, protozoan or helminthic infections, in any location of the body, may be associated with the use of corticosteroids alone or in combination with other immunosuppressive agents that affect cellular immunity, humoral immunity, or neutrophil function.[25]

These infections may be mild, but can be severe and at times fatal. With increasing doses of corticosteroids, the rate of occurrence of infectious complications increases.[26] there may be decreased resistance and inability to localize infection when corticosteroids are used.

Prolonged use of corticosteroids may produce posterior subcapsular cataracts, glaucoma with possible damage to the optic nerves, and may enhance the establishment of secondary ocular infections due to fungi or viruses.

e) Black Fungus Infection (Mucormycosis)

- ✓ Black fungus, also known as Mucormycosis, is a rare but dangerous infection. Black fungus is caused by getting into contact with fungus spores in the environment. It can also form in the skin after the fungus enters through a cut, scrape, burn, or another type of skin trauma.
- ✓ Fungi live in the environment, particularly in soil and decaying organic matter such as leaves, compost piles, rotten wood, and so on. This fungal infection is caused by a type of mould known as 'mucromycetes'. It should be noted that this rare fungal infection affects persons who have health issues or who use drugs that weaken the body's ability to fight the infections. [27]

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i. *Black Fungus Causes*

- ✓ Mucormycetes are a type of mould that causes fungal infections. These moulds can be found everywhere in the environment, including soil, air, and food. They enter the body via the nose, mouth, or eyes and can have an impact on the brain if it is not treated on time. According to medical experts, the main cause of black fungus (mucormycosis) is steroid overuse during COVID treatment.
- ✓ Black fungus (mucormycosis) primarily affects people who have health problems or who take medications that reduce the body's ability to fight germs and illness. The person's immunity is low after covid treatment, which makes them vulnerable to black fungus infection. People with diabetes and COVID-19 patients are at greater risk of developing an infection. [27]

ii. *Black Fungus Risks*

- ✓ People who fall into the following categories are more likely to develop black fungus:
- ✓ Uncontrolled diabetes, diabetic ketoacidosis, and diabetics taking steroids or tocilizumab.
- ✓ Patients taking immunosuppressant's or receiving anticancer treatment, as well as those suffering from a chronic debilitating illness
- ✓ Patients taking high doses of steroids or tocilizumab for an extended period
- ✓ Cases of COVID-19 Severity
- ✓ Patients on oxygen who required nasal prongs, a mask, or a ventilator support
- ✓ Patients who get COVID treatment within six weeks are more likely to develop black fungus.[27]

III. Result and Discussion

A total of 50 doctors and some medical stores from across India were included in the survey. Doctors prescribed more methylprednisolone and dexamethasone drug medicine than steroid medicines to corona patients.

In our research, most side effects were observed for corona patients taking methylprednisolone and dexamethasone drug.

Due to over-prescription of doctors, we came to know from other studies that diabetics who were cured of taking methylprednisolone and dexamethasone medicine when they had corona, got a disease called black fungus after some time. And more deaths from black fungus disease were seen in diabetic patients.

We also found in our survey that Methylprednisolone drug is prescribed more by the doctor in corona patients. Compared to methylprednisolone, doctor prescribed dexamethasone drug is less given in corona patients.

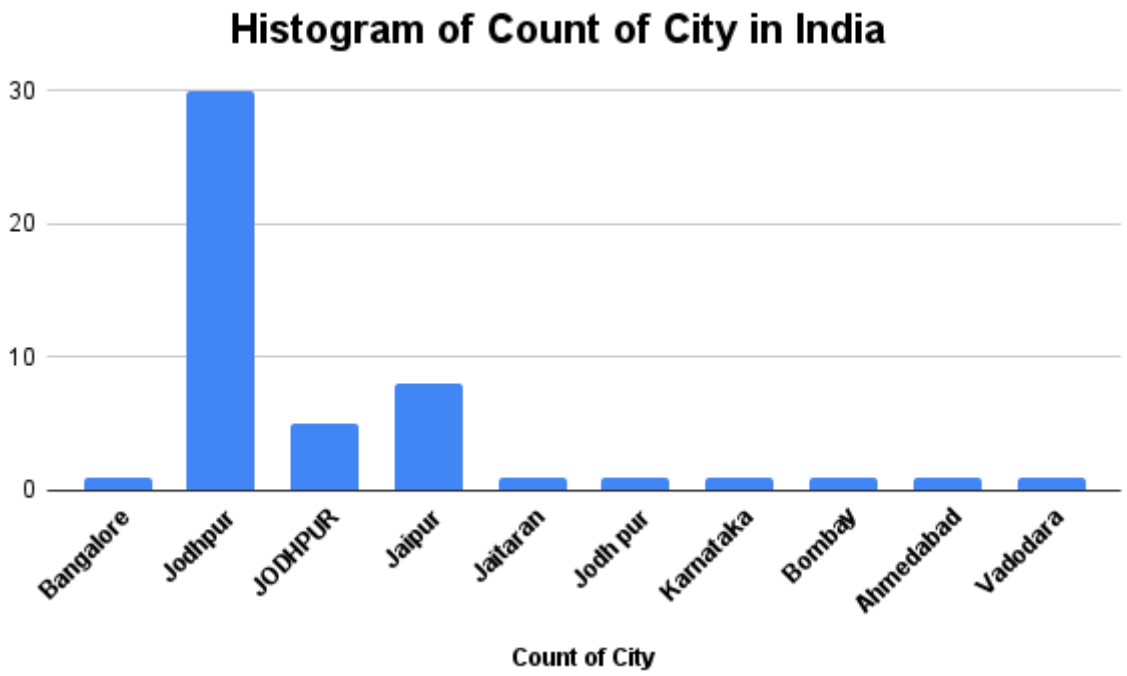


Figure 3: Names of the Cities we Placed in Our Survey

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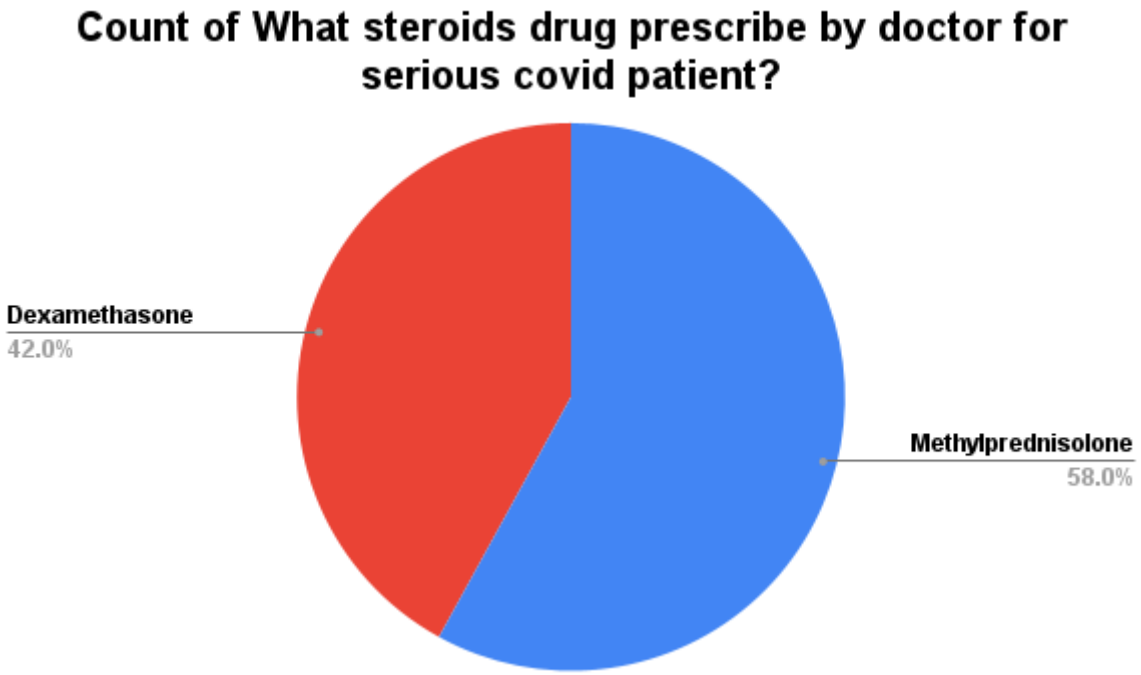


Figure 4: Survey of Which Steroid Drug is More Prescribed by the Doctor in Covid-19 Patients

IV. Conclusion

This research had shown that overdose of methylprednisolone and dexamethasone drug take diabetes patient he has serious eye effect and cause black fungus.

It has been found from research that three things have been detected by giving high amount of steroid drugs to corona patients.

1) Patients who have been cured of corona, who did not have any disease before corona, after recovering from corona in their body, after going home, they have diabetes.
2) Those corona patients who already have diabetes, after being cured by giving more steroid medicine, they got a complaint of black fungus disease.
3) These two things have shown that more steroids are being given to corona patients than giving more side effects because giving more steroids reduces immunity in the body, due to which the black fungus present in the environment is easily found in patients with low immunity. It goes away and the infection increases in the patient's eyes, if the patient does not take treatment on time, then his life is also lost.

However very less work has been on this drug & there is further more scope of scientific investigation.

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Effect of Monoclonal Antibodies Conjugation with Gallium-Containing Solamargine: Warburg Effect-Based Cancer Therapeutic Strategy. Article Review

By Waleed O. Atta
Assiut University

Abstract- Therapy by Monoclonal antibodies is considered extremely hoping method for cancer therapy. But cancer cells have variable methods for resistance by multiple genetic mutations. The aim of that article to illustrate tagging monoclonal antibodies by gallium containing solamargine glycoside within the antibody by glycosylation the asparagine of its Fc portion. Malignant cells need to a big extent high carbohydrate content for aerobic glycolysis for cancer progression. Solamargine as a specific glycoside can be diffused easily and effectively into malignant cells with a high degree of specificity. Complexion gallium to solamargine then conjugation into monoclonal antibodies will increase Monoclonal antibody potency and affinity by Warburg effect based mechanism and gallium particles. Gallium can be retained for a long time inside malignant cells. By that method, the monoclonal antibody will be targeted to cancer cells by solamargine, retained gallium particles besides its functioning specific Fab region.

Keywords: *Fc region, solamargine, gallium, glycosylation, monoclonal antibody, rhamnose.*

GJMR-B Classification: *NLMC Code: QV 704*



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Effect of Monoclonal Antibodies Conjugation with Gallium-Containing Solamargine: Warburg Effect-Based Cancer Therapeutic Strategy. Article Review

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Keywords: Fc region, solamargine, gallium, glycosylation, monoclonal antibody, rhamnose.

I. Introduction

Monoclonal antibodies constitute hoping results for malignancy treatment. Cancer cells have the ability to invade the immune system to predispose invasion and metastasis. Monoclonal antibodies target specific antigens presenting on cancer cells, in a result more specificity for cancer treatment with less side effects. The main role of them is to suppress main checkpoints that are critical for malignancy dissemination such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and tyrosine kinase. Many examples of monoclonal antibodies are produced against specific cancer cell antigens such as antibodies that protects immune checkpoints from cancer cell aggression like lipilimumap, pembrolizumab and alemtuzumab. Also Bevacizumab (anti–VEGF) inhibits the angiogenic activity of VEGF expressing cancer cells. Bevacizumab showed satisfying results in the treatment of metastatic colon cancer, metastatic renal tumors besides non-small cell lung cancer and glioblastoma. Panitumumab (anti-EGFR) is responsible for treatment of metastatic colon cancer expressing epidermal growth factor receptor (EGFR) which has been shown resistance to chemotherapeutic agents. Cetuximab (anti-EGFR) inhibits EGFR related pathways and is used in the treatment of EGFR-positive colon malignant neoplasm and also for head and neck tumors. Ofatumumab showed high efficacy in the treatment chemo-resistant patients with chronic lymphocytic leukemia chemotherapy. Trastuzumab (anti- HER-2/neu) is used in patients with HER-2/neu-positive breast tumor, metastatic gastrointestinal (GI) malignant neoplasms *[Pento, 2017]*. Rituximab has been a cornerstone in treatment of non-Hodgkin's lymphoma, lymphocytic leukemia and other autoimmune diseases such as lupus erythematosus *[Smolej, 2016].* Monoclonal antibodies have been recognized for radioisotopes delivery such as arcitumomab that is a murine antibody fragment that is technetium 99m- labeled. It is a therapeutic agent for patients with metastatic colorectal neoplasm *[Hughes et al, 1997].* Ibritumomab tiuxetan *(fig. 1)* can be tagged with yttrium 90 or Indium 111 which showed high efficacy in treatment of patients with non-Hodgkin's lymphoma and regularity combined with Rituximab *[Rizzieri, 2016].* Tositumomab *(fig.2)* (a MAB labeled) is labeled with iodine 131 used for treatment patients with non-Hodgkin's lymphoma who show bad outcome to other chemotherapeutic drugs *[Shadman et al, 2016].* Also monoclonal antibodies can be labeled by chemotherapeutic agent [chemolabeled antibodies-brentuximab vedotin (Adcetris)]. Another group of monoclonal antibodies are available which is called bispecific mAbs, that has double variable antigen binding fragments (Fabs) whose advantage is to attract cells together. For example, blinatumomab binds CD19 on lymphoma cells and CD3 on T cells, thus prompting T cell cytotoxicity against leukemic B cells *[Goldenberg, 2007].*

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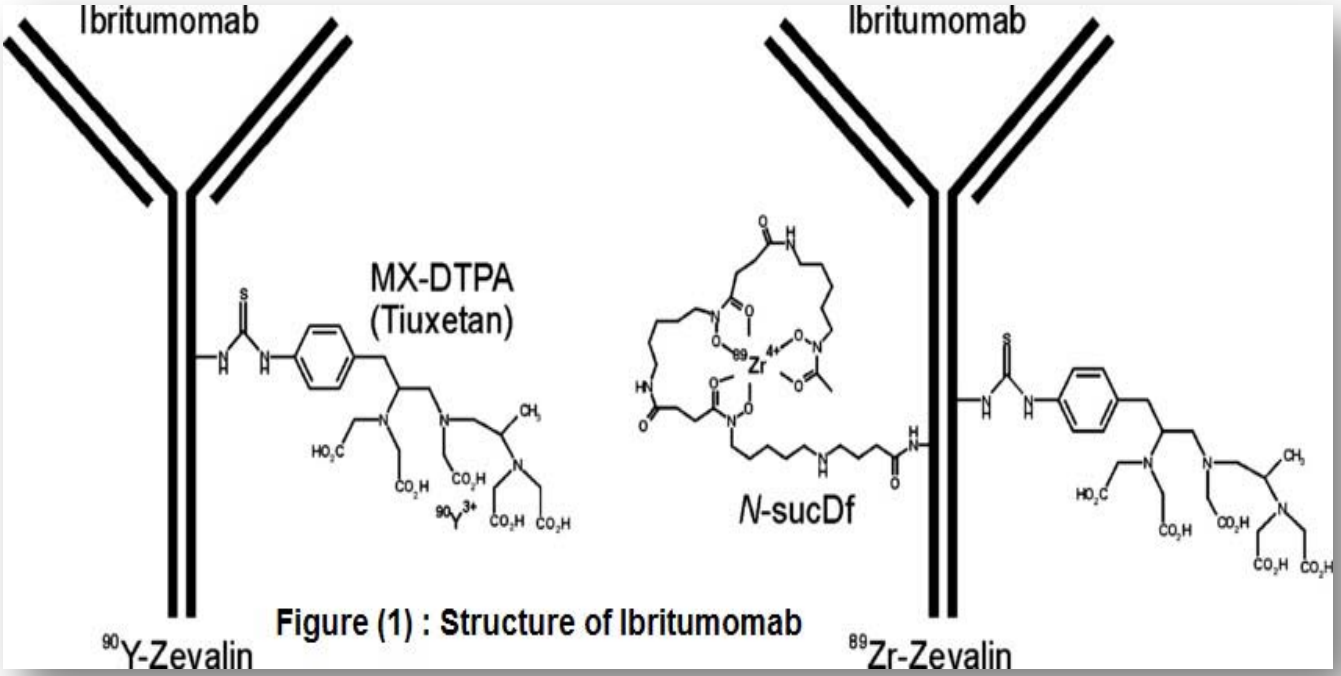


Figure (1): Ibritumomab tiuxetan structure: Tagging Fc portion with yttrium 90 or Indium 111.

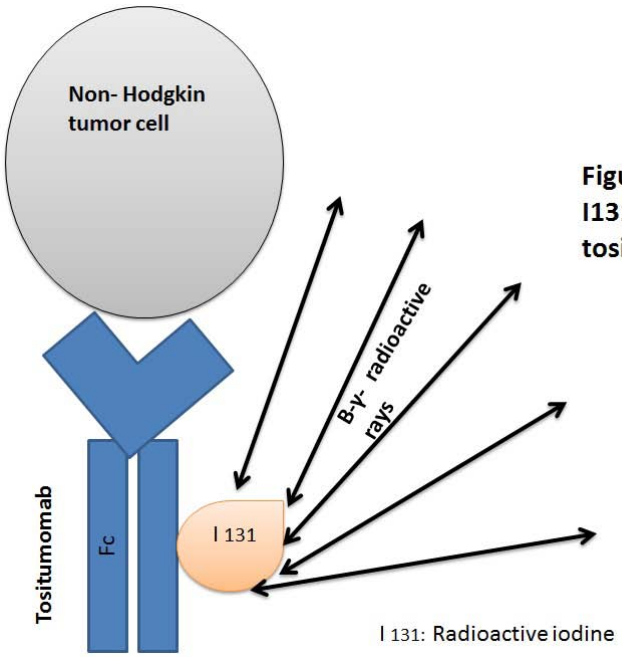


Figure (2) : Radioactive iodine I131 attached to Fc region tositumomab

Figure (2): Tositumomab labeling with iodine 131 used for treatment patients with non-Hodgkin's lymphoma by emission β, γ rays.

II. Structural Insights of Monoclonal Antibody

The general conformation of monoclonal antibody consists of three functional components, two Fragment antigen binding domains (Fabs) and the fragment crystallizable (Fc), with a hinge region between the two Fabs and the Fc that gives the advantage of wide range of flexible mobility to the Fabs. Each of the Fabs contain identical antigen-binding sites that bind with a specific antigen *[Chiu et al, 2019].* The antigen binding sites of antibodies often results in structural variations in the contact surface zones of both the antibody and the antigen. That have been confirmed in the structure studies of both an antibody fragment (Fabs or Fvs) alone and bounded form with its antigen *[Davies*

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and Cohen, 1996]. The Fv region of the Fab consists of a pair of variable domains (VH and VL) together with the HC and LC. In contrast, the glycosylated Fc region binds to variable structures presented on malignant cells and components of the adaptive and humoral immunity. Fc region structure is nearly constant in many human IgG antibodies. It is formed of two constant domains, each one consists of CH2 and CH3. CH3 of both domains are joined tightly together, while CH2s have no protein–protein communication with each other *(fig.3).* The space in-between the CH2s is occupied partially by the carbohydrate attached at Asn297.In some antibodies, the two carbohydrate chains interact through hydrogen bonds or water bringing molecules. The flexibility of the CH2s has its role in the Fc region *[Chiu et al, 2019].* Interaction with Fc gamma receptors (FcR) and the first subcomponent of the C1 complex (C1q) to initiate antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), trogocytosis, stimulation of mediators secretion, and endocytosis of opsonized particles *[Taylor and Lindorfer, 2015].* Now, The Fc region is the target of the developmental engineering for variable effector functions. Structural analysis showed a Fc ball-and-socket joint between CH2 and CH3 that permits the CH2 domain to circulate around its Leu251 side chain, which is buried in a pocket constituted of CH3 residues Met428, His429, Glu430, and His435. FcCH2 contains carbohydrate structures that conceal hydrophobic face of Fc region *[Chiu et al, 2019].* Several Fc glycoform variants and aglycosylated forms have been confirmed such as sialic acids, N-acetylglucosamines, and galactoses, and in some cases, the absence of fucose *[Jefferis, 2005].* Fc glycans improve the antibody biophysical stability *[Lee et al, 2015].* Also they fills the separation distances between CH2. Besides all that they can redirect the effector functionality of the antibody besides changing its the pharmacokinetic profile *[Kronimus et al, 2019].*

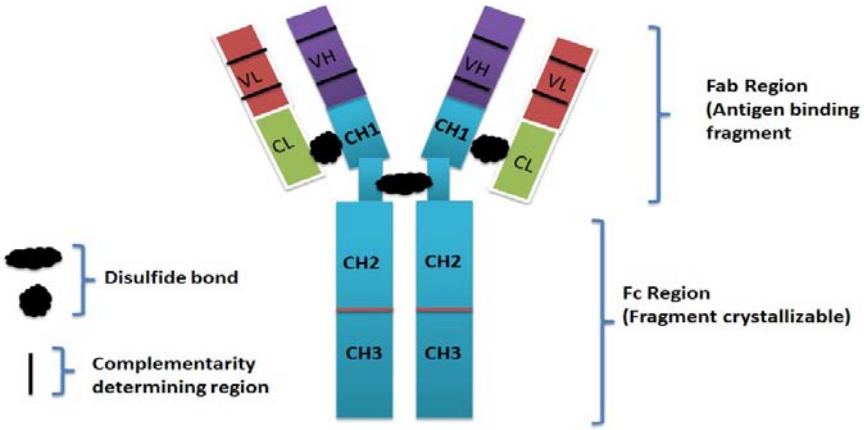


Figure (3): Monoclonal antibody consists of Fab region that contains antigen- binding domain (two variable heavy chains and two variable light chains). Fc region consists of CH2 and CH3. CH2 domain is the glycosylated.

a) *Genetic polymorphism and its effect on monoclonal antibodies efficacy*

Cancer cells have multiple genetic variations that affect monoclonal antibodies efficacy. Genetic polymorphism targets recognition, presentation and metabolism of monoclonal antibodies. Monoclonal antibodies maximum absorption 1-8 days after SC or IM injection *[Korth-Bradley et al, 2000]* and it is determined by blood-tissue hydrostatic gradient besides diffusion through vascular endothelium *[Baxter et al, 1994].* MABs uptake occurs after receptor-mediated endocytosis after binding of Fc domain with FcγR expressed on different immune cells *[Gessner et al, 1998].* However in a recent study, it has been shown that immune system has a necessary role in survival tumor cells that show loss of tumor suppressor genes or activated oncogenes *[Timothy et al, 2021].* Besides that, MABs can bind neonatal Fc receptor (FcRn) at its Fc region (CH2-CH3) domain interface (Ile 253 and two central histidines His 310 and His 435. FcRn protects MAB from intracellular degradation through intracellular recycling mechanisms *[Pyzik et al, 2019].* Reduced expression of FcRn by genetic mutation leads to decreased serum level of MAB and increased clearance ratio *[Ryman and Meibohm, 2017].*

Genetic mutations of cancer cells have inhibitory results on MAB efficacy. For example, BRAFV*600*E, PI3K/ m TOR and PI3K CA genetic mutations expressing colorectal cancer cell lines are associated with low cetuximab and panitumumab efficacy in colorectal cancer treatment potency *[Xu et al, 2017].* Patients that show RAS gene KRAS G*12* A/V

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mutation that upregulates VEGF show lower PFS and OS after treatment with bevacizumab (anti-VEGF) compared to wild type KRAS *[Nakayama et al, 2017]*.

SNPs (single nucleotide polymorphisms) within the PD-L1 gene CD274 have been demonstrated to affect patient improvement to the anti-PD-1 mAb nivolumab. Patients with non-small cell lung cancer that administrated nivolumab possessing the CD274 rs4143815 C/C and C/G genotypes had slightly more elevated median PFS in comparison to patients with the G/G genotype (P = 0.044). Also several studies suggested that PD-L1 rs4143815, that is situated in the 3' untranslated region (UTR) can affect the expression of PD-L1, in a result tumor cells can escape from immune system *[Yeo et 2017]*. Especially, it has been proven that the C allele of rs4143815 has an essential role in an increased production of PD-L1 by attenuating miR-570 *[Wang et al, 2013]*. Also it is clear that patients with the rs4143815 C/C genotype have lower clinical result to paclitaxel and cisplatin chemotherapy *[Lee et al, 2016]*. In addition, during the haplotype analysis, that included seven SNPs (rs733618, rs4553808, rs11571317, rs5742909, rs231775, rs3087243 and rs7565213) within CTLA4 gene, it can be associated with no response to anti-CTLA-4 treatment *[Breunis et al, 2008]*.

b) Value of monoclonal antibodies conjugation with solamargine-gallium containing saccharide

i. *Value of solamargine*

1. Glycoside nature of solamargine: tumor cells have increased needs of glucose for their high rate of replication and invasion. Malignant cells prefer aerobic glycolysis other than mitochondrial oxidation. Rate of glucose metabolism by aerobic glycosylation is roughly 10 -100 more rapid than that of mitochondrial oxidation *[Locasale and Cantley, 2011]*. Also aerobic glycolysis results in production considerable amount of lactic acid after glucose fermentation in the presence of oxygen and functioning mitochondria which is called "Warburg effect". Lactic acid is important for tumor survival and progression *[Maria et al, 2015]*. Besides that, aerobic glycolysis satisfy cancer cell needs of the high requirement of ATP which is necessary for tumor cells division *[Epstein, et al. 2014]*. Also, aerobic glycolysis is considered major factor for carbon production that is crucial for formation of nucleotides, lipid and protein for cancer anabolism and carcinogenic- associated pathways *[Boroughs and DeBerardinis, 2015]*. Warburg effect is essential for NAD+ regeneration that is important for keeping glycolysis active *[Lunt and Vander Heiden, 2011]*. In tumor microenvironment, glucose supply is limited. So tumor cells, stromal cells and immune cells compete for glucose consumption *[Chang C-H, et al, 2015]*. Also within the tumor, glucose as a nutrient is needed for tumor- infiltrating lymphocytes (TILs) for their effect or functions, also it is needed for cancer cell itself. Warburg effect through high aerobic glycolysis within tumor cells compensate TILs for glucose needs *[Chang C-H, et al, 2015]*. Warburg effect has unforgettable role in oncogene mutations such as KRAS in pancreatic cancer and BRAF in melanoma *[Shain AH, et al, 2015]*. From all previous advantages of raid aerobic glycolysis and Warburg effect, we can say that tagging monoclonal antibodies glycosides Fc region with solamargine (glycoside which has high affinity for cancer cells) can be attracting factor for malignant as it can be a glucose supply for their Warburg effect, especially within the low glucose nutrient in the tumor microenvironment (author).
2. Solamargine anti-cancer properties: solamargine is considered one of the glycoalkaloid class (solasodine rhamnosyl glycosides) which shows positive immune response to cancer cells. Solasodine rhamnosyl glycosides are secondary metabolites of plants. They consist of a mono or oligosaccharide chain attached at the C3 position of the nitrogenous steroid alkaloid backbone *[Bill, 2013]*.

Solamargine (SM) molecular formula is C45H73NO15 with the mass of 868.04 Da. Its systematic name is (22R, 25R)- spiro-5-ene-3ß-yl-α-L-rhamnopyranosyl-(1-2glu)-0-α-L-rhamnopyranosyl-(1-4glu)-ß-D-glucopyranose *(fig.4)*. It is chosen from solasodine glycosides because it contains D-glucopyranose which can be conjugated with gallium particles (discussed later.) Solamargine uptake by endogenous endocytic lectins (EELs) expressed on malignant cells results in cellular shrinkage and lysis *[Bill, 2013]*.

The 2 rhamnose moiety of SM have a necessary function in initiation cell death by apoptosis and cytotoxic effects such as human hepatocytes (Hep3B) *[Nakamura et al, 1996]*. It was observed that the carbohydrate moieties of steroidal alkaloids augmented the binding specificity to steroid-associated receptors *[Chang et al, 1998]*. The trisaccharide of SM (two rhamnose units are bound to a glucose moiety), has more affinity to specific cell receptor sites than the corresponding trisaccharide of solasonine (SS) (one rhamnose and one glucose units are connected by a galactose monosaccharide) *[Bill, 2013]*.

Solamargine has potent multiple anti-tumor properties. It showed efficient results in MDR (multiple drug resistance) tumor cells. Solamargine has shown high potency by apoptosis induction in Ehrlich Carcinoma, Leukemia (K562), Colon Cancer (HT-29, HCT-15), Liver Cancer (HepG2, PLC/PRF/5, SMMC-7721), Lung Cancer (A549), Gastric Carcinoma (AGS), Pancreatic Carcinoma (MIA, PaCa-2), Renal Adenocarcinoma (786-0) Uterine Adenocarcinoma

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(HeLa 229), Ovarian Carcinoma (JAM), Mesothelioma (NO36),Glioblastoma, Astrocytoma (U87-MG), Prostate Carcinoma (DV-145, LNCap, PC-3), Melanoma (A2058), Breast Cancer (T47D, MDA-MB-231), Osteosarcoma (U20S) and Squamous Cell Carcinoma (A431, SCC4, SCC9, SCC25). Solamargine also showed selectivity as it did not induce apoptosis in normal cells such as bone marrow cells, fibroblasts, normal hepatocyte cells HL7702 and H9C2 *[Bill, 2013].*

The gene expression of TNFR1 was markedly increased by SM which contributes to the mechanism of the cytotoxicity of SM *[Hsu et al, 1996].* Solamargine triggers the intrinsic and extrinsic pathway of apoptosis in lung and breast cancer cells. SM increased the expressions of external death receptors, such as tumor necrosis factor receptor 1 (TNFR-1), Fas receptor, TNFR-1-associated death domain (TRADD) and Fas-associated death domain (FADD). SM also upregulated the intrinsic ratio of Bax to Bcl-2 by increasing Bax and reduction Bcl-2 and Bcl-xl expressions. As consequence, mitochondrial cytochrome c was released and activation of Caspase-8, -9 and -3 *[Bill, 2013].* Also, SM increased Fas expression and reduced the level of expressed HER2 receptor leading to increased sensitivity of trastuzumab and ant chemotherapy- induced apoptosis in NSCLC A549 and H441 cells, besides breast cancer cells *[Shiu et al, 2009].* Oncosis occurred at high doses of SM in the form of considerable conformations in cell shape and volume, blebs appearance on the cell membranes, mitochondrial swelling, clamping the nuclear contents and finally cell death. Apotosis occurs by low concentrations of SM and both types of cell death (oncosis and apoptosis) were resulted by intermediate concentrations of SM *[Sun et al, 2010].*

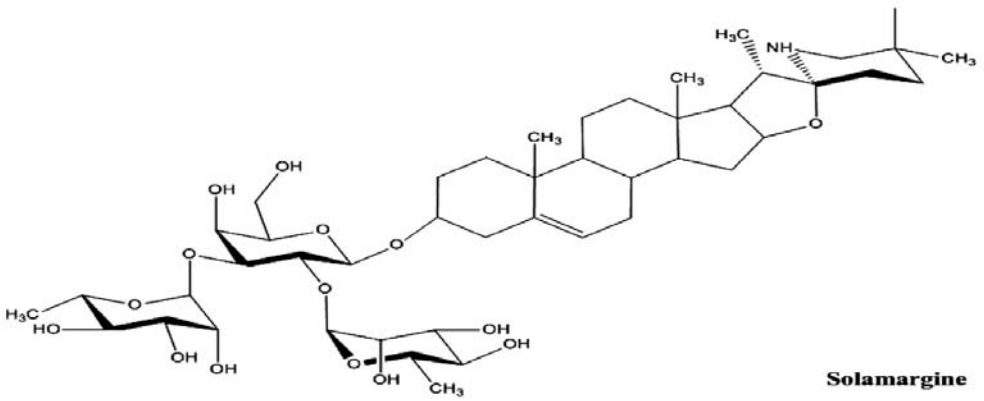


Figure (4): Structure of solamargine

3. Advantages of glycosylated antibodies: The main conserved glycosylation site of IgG is within the Fc region (Asn 297). Types of glycosides attached to Fc region of the monoclonal body determine the efficacy of monoclonal antibody. Antibody-dependent cell cytotoxicity (ADC in no- or low fucosylated MAB is stronger than highly fucosylated MAB. ADCC assay showed that monosialylation of IgG1 gave the advantage of Anti- D Mab for more cell lysis *[Kumpel et al, 1994].* Oligomannose intermediate terminal glycosylation decreased the potency of MAB four-six folds, while the exposed oligomannose structure fastens the monoclonal antibody clearance and reduces its concentration with the patient serum by binding with the mannose receptor on macrophages and phagocytic cells. Degree of galactosylation affects the strength of MAB related ADCC proportionally. In other words, hyper-galactosylation is considered a stimulator for FcγRIIIA– mediated ADCC but does not change the ability of MAB for constitution rosettes with cells that express high-affinity activating FcγRI (associated with myeloid cells) *[Kumpel et al, 1994].* In the opposite, hypogalactosylation results in weak activity of IgG in ADCC. IVIG (intravenous immunoglobulins) attained their efficacy by binding of it Fc region with FcγR-bearing host immune cells *[Galeotti et al, 2009].* That effect can be because of initiation secondary cellular events, like FcγR-induced apoptosis or anergy, including the phosphorylation of immunoreceptor tyrosine-based inhibition motif (ITIM) and immunoreceptor tyrosine-based activation motif (ITAM) *[Hamerman and Lanier, 2006, Siragam et al, 2006].* Therapeutic mAbs demand the

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presence of functioning Fc region to suppress tumor invasion and to raise survival rates in mouse models. Thus, because glycosylation is an essential factor for the functions of human IgG , now, new strategy is adopting conjugation MAB with certain efficient glycoforms for more positive results *[Clynes et al, 1998].*

In my study, solamargine will be conjugated to the glycosylated Fc portion from its steroidal backbone, so that its functioning rhamnose terminal end is free and will be bound to gallium particles (discussed later in the methodology). By that way monoclonal antibody will act directly on malignant cells facilitating the Cytotoxic T cell function. Cancer has the ability to resist monoclonal antibodies by genetic polymorphism (discussed before) and suppression of host immunity (cytotoxic T cells). Cancer immunosuppression is triggered by tumor-derived soluble factors (TDSFs), like interleukin-10 (IL-10), transforming growth factor-b (TGF-b) and vascular endothelial growth factor (VEGF), and that spreads, starting from the primary tumour site reaching to secondary lymphoid organs and peripheral vascularity *[Zou, 2005, Yang, 2004].* Tumor derived VEGF is considered a powerful chemoattractant that initiates migration of immature myeloid cells (iMCs) from the bone marrow into peripheral vessels, where they are attracted to the primary tumor site by the action of chemokines and chemokine receptors *[Kusmartsev and Gabrilovich, 2002].* The iMCs, that entail immature dendritic cells (iDCs) and macrophages, have functional and biochemical remodelling within the tumor microenvironment into tumor-associated iDCs (TiDCs) and tumor- associated macrophages (TAMs) that are recruited to regional lymph nodes, spleen and peripheral circulation for immune evasion. The immunosuppressive iMCs and increased level of reactive oxygen species (ROS) suppress T-cell activation by specific tumor mechanism *[Kusmartsev et al, 2004].* Also the deficient clearance of apoptotic cells triggers formation of anti-DNA-antibodies creating pseudo- autoimmune response against host antigens. In a result pro-inflammatory response appears that increases tumor progression *[Kim et al, 2005].* High levels of auto-antibodies and iDCs stimulate production of CD4+ CD25+ regulatory T cells (Tregs) that suppress T-cell function. iMCs induce their immunosuppressive effect by stimulation of indoleamine 2,3-dioxygenase (IDO) (an enzyme responsible for tryptophan metabolism, tryptophan is needed for T-cell proliferation *[Munn et al 1999]* and ArgI (an enzyme responsible for L-arginine metabolism to ornithine and urea, and the polyamine oxidation from ornithine inhibits IL-2 production, that in a result suppresses T-cell proliferation *[Flescher et al, 1989]* by the help of IL-10 and TGF-b. The final result is production (ROS) that reduce the proliferation of T-cells *[Zea et al, 2005].*

c) *Role of gallium compound (within the solamargine–gallium compound)*

Gallium was chosen due to its role in tumor inhibition besides increased bioavailability and efficacy. Also prolonged presence of gallium intracellular raises its cytotoxicity level *[Rasey et al, 1982].* Selectivity for malignant cells is one of gallium advantages. Ga atoms have the ability to combine to DNA phosphate, constituting a stable complex. Ga compete with magnesium for DNA binding especially affinity of Ga for DNA is 100 times more than of magnesium *[Manfait and Collery 1984]* Ga forms transferrin- Ga complex after favorable binding with transferrin that results in DNA synthesis inhibition by its action on ribonucleotide reductase *[Chitambar et al, 1988].* Ga suppresses biosynthesis pathways within the cell and suppress protein synthesis *[Aoki et al, 1990].* The impact of Ga in affection of cell membrane permeability could be explained by changing the cell membrane potential, modulationof electric charges at the protein synthesis *[Collery et al, 1994].* Ga triggers efflux of calcium from mitochondria which is a necessary starting step for apoptosis *[Gogvadze et al, 1996].* Ga triggers the collagen and fibronectin synthesis *[Bockman et al, 1993]* which might illuminate the cause of the tumor fibrosis after long term administration *[Collery et al, 1986].* Ga is involved in intracellular oxidative stress, with a reduction in the ratio of cellular glutathione reduced form (GSH) on glutathione oxidized form (GSSG), an elevation in metallothionein (MT) and in hemeoxygenase-1 (HO-1) gene expression *[Yang and Chitambar, 2008].* Gallium salicylate *(fig.5)* (tetrakis(1-octanol) *tris*(5-aminosalicylate) gallium(III)) *[Ismail et al, 2006]* has anti-inflammatory, antitumor *[Perugini et al, 2000]* and antiangiogenic characteristics *[Borthwick et al, 2006]* besides the ability to suppress cancer cell progression *[Murono et al, 2000].* Salicylates can reduce platinum- based drugs toxicity *[Li et al, 2002]* and the irradiation toxicity *[Soderberg et al, 1988],* and it can increase chemosensitivity to anticancer drugs *[McCarty and Block, 2006].*

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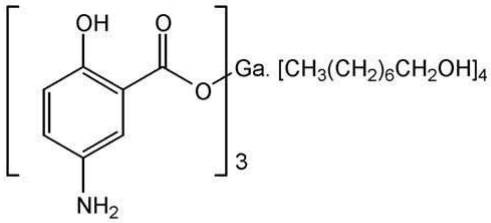


Figure (5): Structure of tetrakis(1-octanol) tris(5-aminosalicylate) gallium(III)

d) Brief illustration of solamargine-gallium advantages when conjugated to monoclonal antibody

1- Increased uptake by malignant cells due high the glycoside content (Warburg- effect base mechanism)
2- Solamargine anti-cancer properties: selectivity and cytotoxicity especially the MAB will bind to solamargine within its steroidal backbone allowing the rhamnose moiety facing cancer cells.
3- Monoclonal antibody glycosylation: solamargine attached to monoclonal antibody will direct it to malignant cell directly facilitating targeting cytotoxic T cells to cancer cells. Also solamargine addition site (Asn 297) (the same site of attachment monoclonal antibody with T cells) will not change the flexibility of Monoclonal antibody. In other words the hinge region of MAB can move with flexibility to attach Solamargine glycoside on tumor cells directly. Also receptor-mediated endocytosis of the modified MAB can occur by binding MAB Fc portion to FcγR expressed on malignant cells. It was observed by flow cytometry, polymerase chain reaction and sequence analysis that FcγR is expressed on malignant cells. Malignant cell FcγR can form complexes with tumor shed antigens and anti-shed tumor antibodies that augment cancer cell proliferation. So we can conclude that modified MAB has competitive antagonism role with tumor shed antigens and anti-shed tumor antibodies on binding to malignant cell FcγR *(Nelson et al, 2001).*
4- There will be three targeting effect or domains within the MAB –(solamargine-Ga) compound. The first is the Fab region of monoclonal antibody itself that antagonizes tumor signaling pathways, the second is solamargine and the third is gallium atoms (solamargine salicylate) that will be bound via its octanol domain to solamargine rhamnose moiety using almond β-glucosidase facing cancer cells.
5- Also the immunosuppressive mechanism of tumor can be compensated by the presence of both gallium and solamargine.
6- Anti-cancer properties of gallium atoms as discussed before.
7- Easy follow up for the tumor size: binding gallium atoms within the cancer will facilitate follow up imaging because gallium used in PTEN scan *[Mikuš et al, 2014].*

e) Chemical and biochemical steps for formation monoclonal antibody conjugated with gallium-containing solamargine

1. *Oxidation of methyl β D-glucoside* Methyl β-d-glucoside, one of the short-chain alkyl glucosides, has been used to synthesize long-chain alkyl glucosides by transacetalization *[Rather et al, 2012].* That reaction can be done by computer modeling with the GRI-Mech 1,2 reaction mechanism and theoretical calculation by the help of the RRKM master equation formalism *[C, -L Yu et al, 1995].* The yield of that reaction will be CH_2O β D-glucoside. *(Reaction 1)*

Reaction (1): oxidation of methyl β D-glucoside

2. *Binding CH2O beta D-glucoside to steroid backbone of solamargine (ethanol) by dehydration reaction* (removal water molecule; OH from CH2O beta D- glucoside and (H) atom from the ethanol group of solamargine with the help of sulferic acid) to form D- glucose-CH-CH2-steroidal backbone of *solamargine*-Rhamnose moiety *(reaction2).* The aim of that reaction to yield solamargine with rhamnose free group, also bound to glucose on its steroidal backbone.

Reaction (2): reaction between solamargine and CH_2O β D- glucoside by dehydratase enzyme to form D-glucose-CH-CH_2-steroidal backbone of solamargine-Rhamnose moiety

3. *Monoclonal antibodies production and glycosylation using CHO-S cells culture*

By the help of PiggyBac (PB) as transposon to carry out the integration of transgenes into the mammalian cells genome *[Wilson et al, 2007].* The PB transposon system can be of one or more transposon donor vectors, that express the transgene(s) and a helper vector encoding the PBase enzyme *[Balasubramanian et al, 2015].* The pB513B1 donor vector and pB200A helper vector will be brought. For construction a dual promoter vector, LC and BGH polyA sequences will be PCR amplified from the pUC-LC and pTracer-CMV2 vectors respectively. After cloning -into an intermediate vector, they will be cloned into pB513B1 by the help of EcoRI/ BamHI enzymes, and pBLP vector

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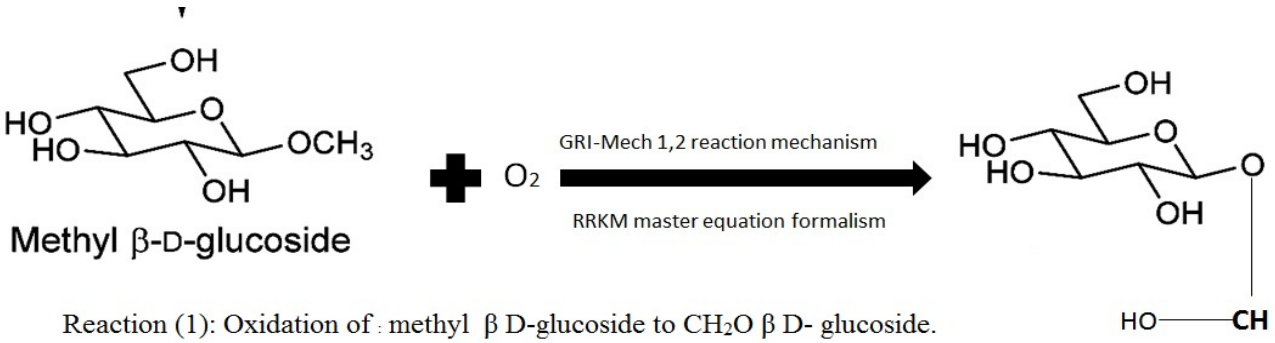
will be obtained. CMV-HC sequence will be sub-cloned from the pTracer-HC vector, into the pBPL vector by BglII/NotI to yield pBLPCH final construct. LC-IRES-HC and LC-F2A-HC (F2A; furin-containing 2A peptide sequence) fragments. LC-IRES-HC containing vector will be digested with NheI/NotI and the resulted fragment attached to the pB513B1 to produce pBLIH donor vector. LC-F2A-HC- cloned into pB513B1 by XbaI/NotI enzymes and pBL2AH will be resulted *[Ahmadi et al 2017].* Also another vector containing amplified sequence of glucosyltransferase enzyme will be prepared and injected into culture media cells. Then using suspension adopted CHO-S cells culture and (by the regular conditions and steps for purification) *[Ahmadi et al 2017]* and solamargine-glucose (formed in step 2), glycosylated monoclonal antibodies will be produced (rhamnose moiety is still not bound) *(reaction 3).*

Reaction (3): Purification of glycosylated monoclonal antibodies

4. *Formation the final form (Monoclonal antibody-solamargine gallium salicylate)* tetrakis (1-octanol) tris (5-aminosalicylate) gallium(III) is the target gallium compound. Its octanol component will be reacted with D-glucopyranose of solamargine rhamnose moiety within the purified glycosylated monoclonal antibody by almond β-glucosidase enzyme (reaction 4). *(Mladenoska, 2016)* The substrates tetrakis (1-octanol) tris(5-aminosalicylate) gallium (III) and the purified glycosylated monoclonal antibody will be dissolved in octanol (dried with 3 Å molecule sieves) to a concentration of 10 mmol/L. The reactions will be done in closed glass vials, in an oven at 50 °C (IgG is denaturated irreversibly at temperature higher than 65° *[Akazawa-Ogawa et 2018]* and completely loses its antigen-binding activity after heat treatment for several minutes at 90 ° *[Akazawa-Ogawa et al, 2014]*, with vigorous shaking at 800 rpm. At different reaction times, samples will be withdrawn from the reaction mixture, put in the freezer for 30 min (monocloanal antibody can be stored in -20 ° without affecting its function *[Johnson, 2012],* diluted with mobile phase, and analyzed by HPLC and spectrophotometer.

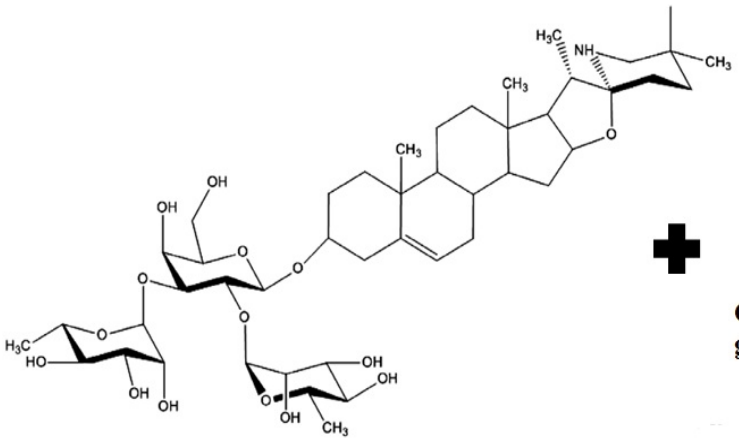
Reaction (4): Synthesis of gallium octyl β-glucoside (glucoside: solamargine glucosyl monoclonal antibody).

Then the efficacy of the modified monoclonal antibody can be compared with the original form of the same monoclonal antibody type, for example comparing cetuximab solamargine –gallium MAB with the efficacy of Cetuximab on epidermal growth factor expressing cancer cell lines such as colorectal cancer cell lines Cac0-2, DLD-1, HCT116 and HT-29.

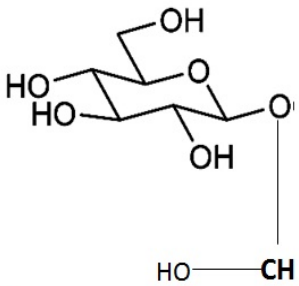


Reaction (1): Oxidation of : methyl β D-glucoside to CH_2O β D- glucoside.

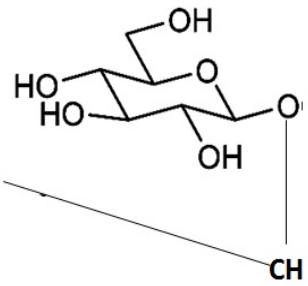
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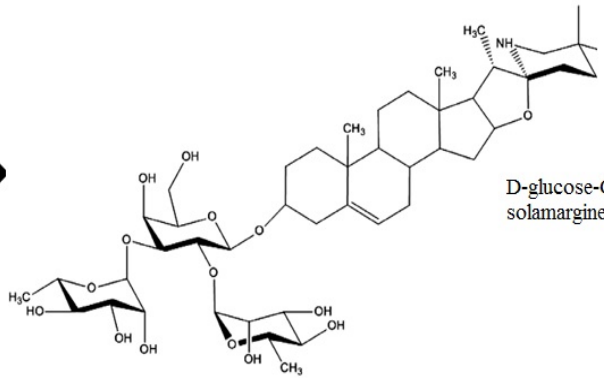
Solamargine



CH_2O β D-glucoside



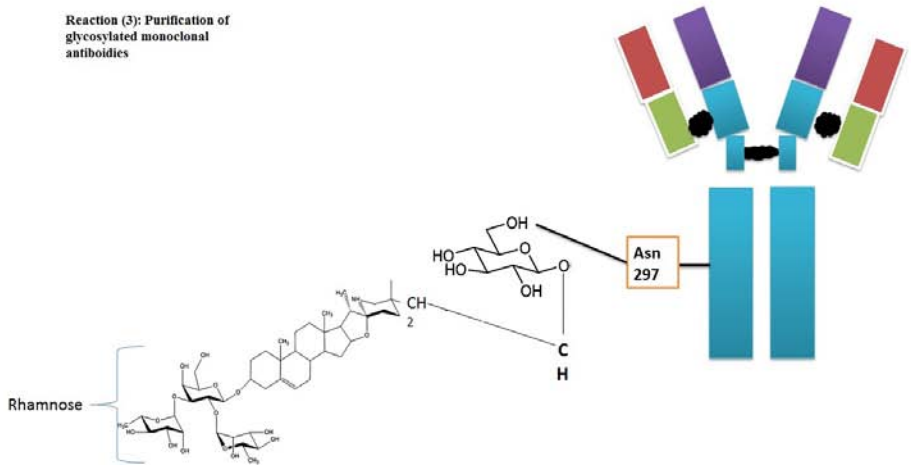
Dehydratase enzyme

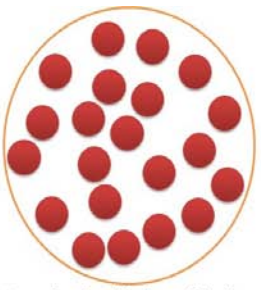


D-glucose-CH-CH_2-steroidal backbone of solamargine-Rhamnose

Reaction (2)

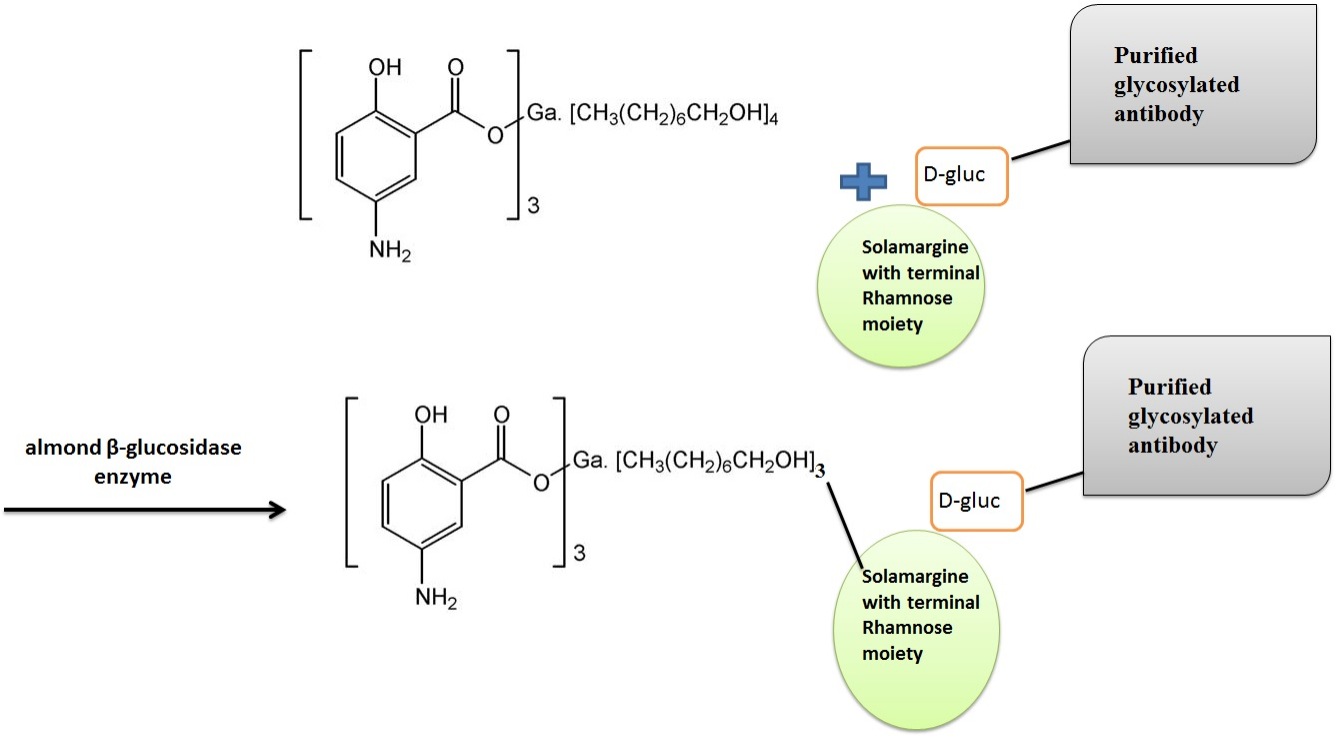
Reaction (3): Purification of glycosylated monoclonal antiboidies





Monoclonal antibody purification using CHO cell and glycosylation with glucose residue of D-glucose-CH-CH_2-steroidal backbone of solamargine-Rhamnose moiety by glucosyltransferase

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III. Conclusion

Modification of monoclonal antibody with gallium containing solamargine can be a general modification to different types of monoclonal antibodies because it is conjugated on Asn 297 which is a fixed structure to all monoclonal antibodies. That modified form can be easily targeted to cancer cells then endocytosis occurs after binding to malignant cell FcγR. Also inhibition the signaling pathway by the action of MAB Fab region will facilitate the suppressive effect of both gallium and solamargine. Besides that, Fab region of MAB can be a targeting structure to direct solamargine and gallium towards tumor cells. On the other side, cancer cells will be suppressed by the modified form of MAB by three components in the same time, MAB itself, gallium and solamargine. By that way, tumor resistance even by genetic polymorphism or immunosuppression of T cells will be markedly affected by the modified MAB if compared to the unmodified one.

Conflict of Interest

Author declares no conflict of interest about the article review.

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14. Arrangement of information: Each section of the main body should start with an opening sentence, and there should be a changeover at the end of the section. Give only valid and powerful arguments for your topic. You may also maintain your arguments with records.

15. Never start at the last minute: Always allow enough time for research work. Leaving everything to the last minute will degrade your paper and spoil your work.

16. Multitasking in research is not good: Doing several things at the same time is a bad habit in the case of research activity. Research is an area where everything has a particular time slot. Divide your research work into parts, and do a particular part in a particular time slot.

17. Never copy others' work: Never copy others' work and give it your name because if the evaluator has seen it anywhere, you will be in trouble. Take proper rest and food: No matter how many hours you spend on your research activity, if you are not taking care of your health, then all your efforts will have been in vain. For quality research, take proper rest and food.

18. Go to seminars: Attend seminars if the topic is relevant to your research area. Utilize all your resources.

19. Refresh your mind after intervals: Try to give your mind a rest by listening to soft music or sleeping in intervals. This will also improve your memory. Acquire colleagues: Always try to acquire colleagues. No matter how sharp you are, if you acquire colleagues, they can give you ideas which will be helpful to your research.

20. Think technically: Always think technically. If anything happens, search for its reasons, benefits, and demerits. Think and then print: When you go to print your paper, check that tables are not split, headings are not detached from their descriptions, and page sequence is maintained.

21. Adding unnecessary information: Do not add unnecessary information like "I have used MS Excel to draw graphs." Irrelevant and inappropriate material is superfluous. Foreign terminology and phrases are not apropos. One should never take a broad view. Analogy is like feathers on a snake. Use words properly, regardless of how others use them. Remove quotations. Puns are for kids, not grunt readers. Never oversimplify: When adding material to your research paper, never go for oversimplification; this will definitely irritate the evaluator. Be specific. Never use rhythmic redundancies. Contractions shouldn't be used in a research paper. Comparisons are as terrible as clichés. Give up ampersands, abbreviations, and so on. Remove commas that are not necessary. Parenthetical words should be between brackets or commas. Understatement is always the best way to put forward earth-shaking thoughts. Give a detailed literary review.

22. Report concluded results: Use concluded results. From raw data, filter the results, and then conclude your studies based on measurements and observations taken. An appropriate number of decimal places should be used. Parenthetical remarks are prohibited here. Proofread carefully at the final stage. At the end, give an outline to your arguments. Spot perspectives of further study of the subject. Justify your conclusion at the bottom sufficiently, which will probably include examples.

23. Upon conclusion: Once you have concluded your research, the next most important step is to present your findings. Presentation is extremely important as it is the definite medium though which your research is going to be in print for the rest of the crowd. Care should be taken to categorize your thoughts well and present them in a logical and neat manner. A good quality research paper format is essential because it serves to highlight your research paper and bring to light all necessary aspects of your research.

Informal Guidelines of Research Paper Writing

Key points to remember:

- Submit all work in its final form.
- Write your paper in the form which is presented in the guidelines using the template.
- Please note the criteria peer reviewers will use for grading the final paper.

Final points:

One purpose of organizing a research paper is to let people interpret your efforts selectively. The journal requires the following sections, submitted in the order listed, with each section starting on a new page:

The introduction: This will be compiled from reference matter and reflect the design processes or outline of basis that directed you to make a study. As you carry out the process of study, the method and process section will be constructed like that. The results segment will show related statistics in nearly sequential order and direct reviewers to similar intellectual paths throughout the data that you gathered to carry out your study.

The discussion section:

This will provide understanding of the data and projections as to the implications of the results. The use of good quality references throughout the paper will give the effort trustworthiness by representing an alertness to prior workings.

Writing a research paper is not an easy job, no matter how trouble-free the actual research or concept. Practice, excellent preparation, and controlled record-keeping are the only means to make straightforward progression.

General style:

Specific editorial column necessities for compliance of a manuscript will always take over from directions in these general guidelines.

To make a paper clear: Adhere to recommended page limits.



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Mistakes to avoid:

- Insertion of a title at the foot of a page with subsequent text on the next page.
- Separating a table, chart, or figure—confine each to a single page.
- Submitting a manuscript with pages out of sequence.
- In every section of your document, use standard writing style, including articles ("a" and "the").
- Keep paying attention to the topic of the paper.
- Use paragraphs to split each significant point (excluding the abstract).
- Align the primary line of each section.
- Present your points in sound order.
- Use present tense to report well-accepted matters.
- Use past tense to describe specific results.
- Do not use familiar wording; don't address the reviewer directly. Don't use slang or superlatives.
- Avoid use of extra pictures—include only those figures essential to presenting results.

Title page:

Choose a revealing title. It should be short and include the name(s) and address(es) of all authors. It should not have acronyms or abbreviations or exceed two printed lines.

Abstract: This summary should be two hundred words or less. It should clearly and briefly explain the key findings reported in the manuscript and must have precise statistics. It should not have acronyms or abbreviations. It should be logical in itself. Do not cite references at this point.

An abstract is a brief, distinct paragraph summary of finished work or work in development. In a minute or less, a reviewer can be taught the foundation behind the study, common approaches to the problem, relevant results, and significant conclusions or new questions.

Write your summary when your paper is completed because how can you write the summary of anything which is not yet written? Wealth of terminology is very essential in abstract. Use comprehensive sentences, and do not sacrifice readability for brevity; you can maintain it succinctly by phrasing sentences so that they provide more than a lone rationale. The author can at this moment go straight to shortening the outcome. Sum up the study with the subsequent elements in any summary. Try to limit the initial two items to no more than one line each.

Reason for writing the article—theory, overall issue, purpose.

- Fundamental goal.
- To-the-point depiction of the research.
- Consequences, including definite statistics—if the consequences are quantitative in nature, account for this; results of any numerical analysis should be reported. Significant conclusions or questions that emerge from the research.

Approach:

- Single section and succinct.
- An outline of the job done is always written in past tense.
- Concentrate on shortening results—limit background information to a verdict or two.
- Exact spelling, clarity of sentences and phrases, and appropriate reporting of quantities (proper units, important statistics) are just as significant in an abstract as they are anywhere else.

Introduction:

The introduction should "introduce" the manuscript. The reviewer should be presented with sufficient background information to be capable of comprehending and calculating the purpose of your study without having to refer to other works. The basis for the study should be offered. Give the most important references, but avoid making a comprehensive appraisal of the topic. Describe the problem visibly. If the problem is not acknowledged in a logical, reasonable way, the reviewer will give no attention to your results. Speak in common terms about techniques used to explain the problem, if needed, but do not present any particulars about the protocols here.

The following approach can create a valuable beginning:

- Explain the value (significance) of the study.
- Defend the model—why did you employ this particular system or method? What is its compensation? Remark upon its appropriateness from an abstract point of view as well as pointing out sensible reasons for using it.
- Present a justification. State your particular theory(-ies) or aim(s), and describe the logic that led you to choose them.
- Briefly explain the study's tentative purpose and how it meets the declared objectives.

Approach:

Use past tense except for when referring to recognized facts. After all, the manuscript will be submitted after the entire job is done. Sort out your thoughts; manufacture one key point for every section. If you make the four points listed above, you will need at least four paragraphs. Present surrounding information only when it is necessary to support a situation. The reviewer does not desire to read everything you know about a topic. Shape the theory specifically—do not take a broad view.

As always, give awareness to spelling, simplicity, and correctness of sentences and phrases.

Procedures (methods and materials):

This part is supposed to be the easiest to carve if you have good skills. A soundly written procedures segment allows a capable scientist to replicate your results. Present precise information about your supplies. The suppliers and clarity of reagents can be helpful bits of information. Present methods in sequential order, but linked methodologies can be grouped as a segment. Be concise when relating the protocols. Attempt to give the least amount of information that would permit another capable scientist to replicate your outcome, but be cautious that vital information is integrated. The use of subheadings is suggested and ought to be synchronized with the results section.

When a technique is used that has been well-described in another section, mention the specific item describing the way, but draw the basic principle while stating the situation. The purpose is to show all particular resources and broad procedures so that another person may use some or all of the methods in one more study or referee the scientific value of your work. It is not to be a step-by-step report of the whole thing you did, nor is a methods section a set of orders.

Materials:

Materials may be reported in part of a section or else they may be recognized along with your measures.

Methods:

- Report the method and not the particulars of each process that engaged the same methodology.
- Describe the method entirely.
- To be succinct, present methods under headings dedicated to specific dealings or groups of measures.
- Simplify—detail how procedures were completed, not how they were performed on a particular day.
- If well-known procedures were used, account for the procedure by name, possibly with a reference, and that's all.

Approach:

It is embarrassing to use vigorous voice when documenting methods without using first person, which would focus the reviewer's interest on the researcher rather than the job. As a result, when writing up the methods, most authors use third person passive voice.

Use standard style in this and every other part of the paper—avoid familiar lists, and use full sentences.

What to keep away from:

- Resources and methods are not a set of information.
- Skip all descriptive information and surroundings—save it for the argument.
- Leave out information that is immaterial to a third party.



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Results:

The principle of a results segment is to present and demonstrate your conclusion. Create this part as entirely objective details of the outcome, and save all understanding for the discussion.

The page length of this segment is set by the sum and types of data to be reported. Use statistics and tables, if suitable, to present consequences most efficiently.

You must clearly differentiate material which would usually be incorporated in a study editorial from any unprocessed data or additional appendix matter that would not be available. In fact, such matters should not be submitted at all except if requested by the instructor.

Content:

- Sum up your conclusions in text and demonstrate them, if suitable, with figures and tables.
- In the manuscript, explain each of your consequences, and point the reader to remarks that are most appropriate.
- Present a background, such as by describing the question that was addressed by creation of an exacting study.
- Explain results of control experiments and give remarks that are not accessible in a prescribed figure or table, if appropriate.
- Examine your data, then prepare the analyzed (transformed) data in the form of a figure (graph), table, or manuscript.

What to stay away from:

- Do not discuss or infer your outcome, report surrounding information, or try to explain anything.
- Do not include raw data or intermediate calculations in a research manuscript.
- Do not present similar data more than once.
- A manuscript should complement any figures or tables, not duplicate information.
- Never confuse figures with tables—there is a difference.

Approach:

As always, use past tense when you submit your results, and put the whole thing in a reasonable order.

Put figures and tables, appropriately numbered, in order at the end of the report.

If you desire, you may place your figures and tables properly within the text of your results section.

Figures and tables:

If you put figures and tables at the end of some details, make certain that they are visibly distinguished from any attached appendix materials, such as raw facts. Whatever the position, each table must be titled, numbered one after the other, and include a heading. All figures and tables must be divided from the text.

Discussion:

The discussion is expected to be the trickiest segment to write. A lot of papers submitted to the journal are discarded based on problems with the discussion. There is no rule for how long an argument should be.

Position your understanding of the outcome visibly to lead the reviewer through your conclusions, and then finish the paper with a summing up of the implications of the study. The purpose here is to offer an understanding of your results and support all of your conclusions, using facts from your research and generally accepted information, if suitable. The implication of results should be fully described.

Infer your data in the conversation in suitable depth. This means that when you clarify an observable fact, you must explain mechanisms that may account for the observation. If your results vary from your prospect, make clear why that may have happened. If your results agree, then explain the theory that the proof supported. It is never suitable to just state that the data approved the prospect, and let it drop at that. Make a decision as to whether each premise is supported or discarded or if you cannot make a conclusion with assurance. Do not just dismiss a study or part of a study as "uncertain."

Research papers are not acknowledged if the work is imperfect. Draw what conclusions you can based upon the results that you have, and take care of the study as a finished work.

- You may propose future guidelines, such as how an experiment might be personalized to accomplish a new idea.
- Give details of all of your remarks as much as possible, focusing on mechanisms.
- Make a decision as to whether the tentative design sufficiently addressed the theory and whether or not it was correctly restricted. Try to present substitute explanations if they are sensible alternatives.
- One piece of research will not counter an overall question, so maintain the large picture in mind. Where do you go next? The best studies unlock new avenues of study. What questions remain?
- Recommendations for detailed papers will offer supplementary suggestions.

Approach:

When you refer to information, differentiate data generated by your own studies from other available information. Present work done by specific persons (including you) in past tense.

Describe generally acknowledged facts and main beliefs in present tense.

The Administration Rules

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Topics	Grades		
	A-B	**C-D**	**E-F**
Abstract	Clear and concise with appropriate content, Correct format. 200 words or below	Unclear summary and no specific data, Incorrect form Above 200 words	No specific data with ambiguous information Above 250 words
Introduction	Containing all background details with clear goal and appropriate details, flow specification, no grammar and spelling mistake, well organized sentence and paragraph, reference cited	Unclear and confusing data, appropriate format, grammar and spelling errors with unorganized matter	Out of place depth and content, hazy format
Methods and Procedures	Clear and to the point with well arranged paragraph, precision and accuracy of facts and figures, well organized subheads	Difficult to comprehend with embarrassed text, too much explanation but completed	Incorrect and unorganized structure with hazy meaning
Result	Well organized, Clear and specific, Correct units with precision, correct data, well structuring of paragraph, no grammar and spelling mistake	Complete and embarrassed text, difficult to comprehend	Irregular format with wrong facts and figures
Discussion	Well organized, meaningful specification, sound conclusion, logical and concise explanation, highly structured paragraph reference cited	Wordy, unclear conclusion, spurious	Conclusion is not cited, unorganized, difficult to comprehend
References	Complete and correct format, well organized	Beside the point, Incomplete	Wrong format and structuring

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