DISCUSSION

There are many plants with ethnomedicinal values. They have been used by human beings for centuries in the treatment of wide variety of diseases. Still, they lack the scientific evidence to justify their folklore claims. *Avicennia alba* is one of these plants, which has not been studied thoroughly in the scientific community. It is mainly found in Sunderban area of West Bengal. The aerial parts of this plant are used as antifertility agents by the people of Rarrh region of West Bengal.

The present study was undertaken to evaluate its antifertility activity in female rats. The aerial parts (stems, leaves, fruits and flowers- 4:3:2:1 by w/w) are defatted and then extracted with methanol by soxhlation. Methanol dissolve chiefly polar metabolities together with medium and low polarity compounds. So, methanolic extract was used in this study. Phytochemical analysis of this extract was also done to identify the chemical constituents present in it. One novel compound was also isolated. Some other biological activities (like analgesic, antipyretic, muscle relaxant, antimicrobial and anti oxidant activity) were also evaluated based on chemical constituents present in the extract. Molecular docking studies on the active principles present in the plant were carried out to correlate the pharmacological activities with active principles.

In the acute toxicity study OECD guideline 420 was followed. In the sighting study no toxicity was found at the dose of 2000 mg/kg. So the starting dose for the main study was chosen as 2000 mg/kg. There was no toxicity in all the 5 animals administered with 2000 mg/kg. So the extract can be classified as 5/unclassified according to the globally harmonized classified system (GHS). Based on this 3 doses of the extract has been selected for this study i.e. 100, 200 and 400 mg/kg body weight.

To evaluate the antifertility activity, the methanolic extract was studied for anti-implantation activity, estrogenic/antiestrogenic activity in female rats. Animals were observed for uterotrophic changes and vaginal cornification. The change in phases of
oestrous cycle (if any) was also studied. Its effect on biochemical parameters like uterine cholesterol content and uterine total protein concentration was also studied to get more evidence on the molecular mechanism. Histological examination of uterine horn was also done.

The methanolic extract of *Avicennia alba* showed 51.06%, 67.37% and 85.77% antiimplantation activity and 78.47%, 86.95% and 97.09% antifertility activity. The equilibrium of secretion of female sex hormones like estrogen and progesterone are essential for implantation and maintenance of pregnancy. Any hormonal imbalance can cause antiimplantation or can induce abortion or may cause infertility. Again a proper estrogen and progesterone balance is required for uterine receptivity to the embryo.

Again the extract also showed significant increase in duration of diestrous phase and decrease in duration of metaestrous phase as compared to control. The loss of normal estrous cycle indicates the disruption of female ovarian hormone estrogen and progesterone balance. So the antiimplantation activity of *Avicennia alba* may be associated with estrogen and progesterone imbalance.

In another study, ovariectomized female rats were used. Estrogen causes various physiologic and biochemical changes in uterus and the female reproductive tract. When female rats are ovariectomized, the resultant lack of estrogen causes atrophy of the uterus. Administration of estrogenic substances to ovariectomized rats causes uterotrophic effects, vaginal cornification and proliferative changes in uterine endometrium epithelium height. Our study revealed the estrogenic activities of methanolic extract of *Avicennia alba* like increase in uterine weight, diameter of uterus, thickness of endometrium and vaginal cornification.

Estrogen like steroid stimulates the synthesis of mRNA, DNA and protein. In the ovariectomized rats, the uterine protein concentration is increased by estrogen. So the increase in uterine total protein concentration in our present study further reveals the presence of estrogen like steroidal substance in methanolic extract of *Avicennia alba*. 
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Cholesterol is a precursor molecule in steroidogenesis. It is previously reported that estrogen administration increase the uterine cholesterol content in ovariectomised rats to stimulate steroidogenesis. The methanolic extract increases the cholesterol level in ovariectomised rat uterus. This again confirms the presence of estrogen like steroidal substances in *Avicennia alba*.

In another study on histological examination of uterus, the rats treated with methanolic extract of *Avicennia alba* showed estrogenic influence. This is evidenced by increase in height of luminal epithelium with loose stroma and increased number of glands.

The degree of uterotrophic potency of the methanolic extract is more than that of Ethinyl estradiol. When both ethinyl estradiol and methanolic extract were combinelly administered there was significant increase in the uterine weight as compared to their individual effects. So the plant does not possess antiestrogenic activity in the presence of ethinyl estradiol. Hence the plant may be beneficial both as a contraceptive and post menopausal hormonal replacement therapy.

Thorough literature review showed the presence of steroids, flavonoids, alkaloids, saponins and tannins in *Avicennia alba*. The GC-MS analysis of the methanolic extract revealed the presence of 10 bioactive compounds. They are Hexadecanoic acid, phytol, 9, 12, 15-octadecatrienoic acid, stigmasterol, squalene etc.

Hexadecanoic acid is known to possess antibacterial, antifungal, Antitumor and anti-inflammatory activity. 9,12,15-Octadecatrienoic acid, methyl ester possesses antimicrobial, analgesic, anesthetic, allergenic, anti-convulsant, anti-inflammatory, antipyretic, antioxidant, hepatoprotective, hypocholesterolemic, nematicide, antisalmonella and antiseptic activities. Squalene was previously reported to have emollient, skin hydration, antibacterial, antioxidant and antitumor activities. Squalene is also a lipooxygenase inhibitor.

There are reports that phytoestrogens like β-sitosterol, Flavonoids and phenolic compounds are known to possess estrogenic activity. Phytoestrogens like β-sitosterol, stigmasterol etc possess estrogenic activity due to their affinity with estrogenic
receptors.\textsuperscript{220, 93} So, the presence of flavonoids and phytosterol may be responsible for its estrogenic activity.

Phytol is a phytosterol that has been claimed to exhibit estrogenic activity due to its affinity towards estrogenic receptors leading to infertility in animals.\textsuperscript{93} Phytanic acid, an active metabolite of phytol has also been reported to activate estrogen responsive genes by activating nuclear receptors like peroxisome proliferator-activated receptors (PPARs) that heterodi-merizes with retinoid X receptor (RXR).\textsuperscript{221, 222} The presence of phytoestrogens like phytol and stigmasterol may be responsible for disruption of estrous cycle and hence anti-implantation activity.

In the molecular docking analysis, phytol possesses binding energy of -5.2 kcal/mol with the estrogenic receptor (PDB ID- 1DHT). This shows the estrogenic activity of phytol. It is reported that estrogenic activity causes expulsion of ova from fallopian tube or disruption of the luteotrophic activity of the blastocyst.\textsuperscript{221} Stigmasterol also possesses binding energy of -7.4 kcal/mol with the estrogenic receptor (PDB ID- 1DHT). Stigmasterol possesses estrogenic activity and have been claimed to have estrogenic activity due to estrogen receptors leading to rat.\textsuperscript{93} So the estrogenic activity of phytoestrogens like phytol and stigmasterol may be responsible for the anti-implantation activity of the methanolic extract of \textit{Avicennia alba}. Stigmasterol may be major contributor for the anti-implantation activity.

It is widely reported that phenolic and flavonoid compounds may significantly contribute to overall antimicrobial activity.\textsuperscript{223} Flavonoids are reported to have anti-inflammatory, antibacterial, antiviral, anticancer, and anti-allergic activities.\textsuperscript{224} Our study revealed the antibacterial and anthelmintic activity of the methanolic extract of \textit{A. alba}. The extract as well as its n-hexane fraction was more effective against \textit{S. aureus}.

The gram-positive bacterial strains were more susceptible to the plant extracts as compared to gram negative bacteria. This is in agreement with previous reports that plant extracts are more active against gram-positive bacteria.\textsuperscript{225}

The antibacterial activity of the plant extract may be attributed due to the presence of bioactive compounds like phenolic compounds and flavonoids.\textsuperscript{226} Among
these bioactive compounds, phenolics were the most important active compounds against bacteria.\textsuperscript{227} Thus the results of antibacterial activities obtained in the present study for the methanolic extract of \textit{A. alba} can be attributed to their total phenolic compounds.

There are also reports of hexadecanoic acid possessing antibacterial activity and 9,12,15-Octadecatrienoic acid possessing nematicide activity.\textsuperscript{213-216} The extract showed anthelmintic activity (against \textit{Pheretima posthuma}) at 10 mg/ml. In our GC MS analysis study hexadecanoic acid and 9, 12,15-Octadecatrienoic acid are found to be present in the methanolic extract of \textit{A. alba}. So the antibacterial activity may be attributed to hexadecanoic acid and anthelmintic activity may be attributed to 9,12,15-Octadecatrienoic acid.

Nowadays, the plants serve as the major source of bioactive compounds for the development of new therapeutic agents and play a vital role in free radical scavenging and antioxidant activity.\textsuperscript{228} Phenolics such as flavonoids and polyphenolic compounds are mostly distributed in the plants. The synthetic antioxidants have limitation for use because of their carcinogenic property. Therefore, the need of searching for natural antioxidants has been increasing vastly in present years.\textsuperscript{229}

The free radicals are generated continuously in the living organism during metabolic processes. The oxygen free radicals comprise singlet oxygen, superoxide, alkyl peroxyl, alkoxyl, and hydroxyl, which are also involved in regulation of cell growth and intercellular signalling. In oxidative stress, free radicals attack lipids in cell membranes, proteins in tissues and enzymes and DNA, which cause membrane damage, protein modifications and DNA damage. These oxidative damages play a causative role in a series of human diseases such as cancer, atherosclerosis, cardiovascular disease, aging and various other disorders.\textsuperscript{230}

\textit{DPPH} is a stable; nitrogen cantered free radical, which produces deep purple colour in methanol solution. The principle of this assay is based on the reduction of purple coloured methanolic \textit{DPPH} solution in the presence of antioxidants by the formation of yellow coloured diphenyl-picryl hydrazine. As absorbance decreases, the antioxidant activity of the extract increases. Our results suggest that different
concentrations have different activities and maximum activity was observed at the concentration of 200 μg/ml. The observed antioxidant of methanolic extract of the plant may be due to the neutralization of free radicals (DPPH), either by transfer of hydrogen atom or by transfer of an electron.\textsuperscript{231}

Superoxide anions can be generated either from NADPH oxidase or as a result of electron leakage from mitochondrial electron transport system. Superoxide anions may react with biological macromolecules leading to various tissue damages.\textsuperscript{232, 233}

Several studies suggest that NO may modulate iron-catalyzed oxidation reactions such as the $O_2^-$ driven Fenton reaction, which produces powerful oxidants such as the hydroxyl radical (·OH) and metalloxo complexes. The mechanisms by which NO may inhibit lipid peroxidation are not clear, however, one possible mechanism of lipid peroxidation inhibition is due to termination of reaction propagation.\textsuperscript{234}

Hydroxyl is a weak oxidizing agent and can inactivate a few enzymes directly, by oxidation of essential thiol (-SH) groups. It rapidly transverses the cell membrane and react with Fe\textsuperscript{2+} and Cu\textsuperscript{2+} ions to form hydroxyl radical. This reaction may be the origin of many of its toxic effects.\textsuperscript{235}

It has been reported previously that the antioxidant activity of phenolics is mainly due to their redox properties, and singlet oxygen quenching. Flavonoids may exhibit antioxidative effects as free radical scavengers, mainly attributed to their phenolic hydroxyl groups attached to ring structures.\textsuperscript{236} Flavonoids are naturally occurring compounds in plants and are thought to have positive effects on human health and their free radical scavenging effect is greater than vitamin C and E.\textsuperscript{237} In the present investigation, methanolic extract of \textit{Avicennia alba} exhibited outstanding scavenging effects on DPPH, Superoxide, nitric oxide and Hydroxyl radicals. The total phenolic and flavonoid content of methanolic exteact of \textit{Avicennia alba} was estimated as 185 mg Gallic acid equivalent/g and 92 mg quercetin equivalent/g respectively. So our results suggested that phenolic compounds and flavonoids found in methanol extract of \textit{A. alba} may be the major contributors for the antioxidant activity.
There are reports of anti-inflammatory activity of *A. alba*.\(^{238}\) Hexadecanoic acid possesses anti-inflammatory effect.\(^{239}\) Octadecatrienoic acid possesses analgesic, antipyretic and anti-inflammatory activity.\(^{216}\) So we have planned to evaluate analgesic and antipyretic activity. Our results revealed significant analgesic and antipyretic activity. The analgesic and antipyretic activity may be due to presence of flavonoids present in the plant extract.\(^{240}\) Since this plant or extract showed analgesic and antipyretic effect, we have then planned to screen its muscle relaxant activities using Rota rod test. The extract showed significant muscle relaxant activity. Muscle relaxant activity of the plant extract might be attributed the presence of Hexadecanoic acid and Octadecatrienoic acid.\(^{241}\)

A dark yellow compound was isolated from n-hexane–ethyl acetate (7:3) eluants of methanolic extract of *Avicennia alba* with melting point of 150-160°C. The structure was confirmed by comparison of its different spectral data with the corresponding authentic drug or literature based data. The UV absorption (\(\lambda_{\text{max}}\): 228, 312nm in MeOH) exhibited the characteristic absorption maxima for a flavonoid and further chemically elucidated by addition of few drops of ceric ammonium nitrate reagent. It produced red coloration suggesting the presence of alcoholic groups.\(^{243}\)

The FTIR spectroscopic analysis exhibited characteristic broad peak centered at 3154.97 cm\(^{-1}\) that is characteristic of O-H stretching suggesting the presence of hydroxyl group which was substantiated by chemical identification test. The absorption bands at 2853.48 corresponds to CH\(_2\)- stretching, at 1710.56 cm\(^{-1}\) as a result of C=O stretching, at 1610.27 cm\(^{-1}\) as a result of CH=CH stretching, absorption band at 1175.40 cm\(^{-1}\) is due to the presence of C-O-C stretching. The structure of isolated flavone was elucidated by \(^1^H\) NMR and \(^{13}\)C-NMR techniques as by comparison of spectral data with that of reported earlier.\(^{244}\)

The \(^{13}\)C-NMR spectrum of flavone revealed the presence of approximately 21 peaks; twelve aromatic carbon, four active methylene, two methoxy and \(\alpha\)-\(\beta\) unsaturated hydrocarbon atom attached to ketone system. The presence of aromatic hydrocarbon of the flavone were structurally proved by sharp signal at 125.9, 123.9, 135.1, 116.1,156.4, 123.8, 109.5, 154.6,115.1,131.9,128.4 and 126.5 which were
attributed to the position of (C-5), (C-6), (C-7), (C-8), (C-9), (C-10), (C-1'), (C-2'), (C-3'), (C-4'), (C-5') and (C-6') respectively. The absorption signal at 64.0 ppm, due to the presence of O-H attached to the carbon at C-3'' and also exhibited characteristic broad peak at 3154.97 cm\(^{-1}\) appeared in FTIR spectrum.

The \(^{13}\text{C}\)-NMR showed most recognizable down field sharp signal at \(\delta\) 177.5 ppm, corresponds to the presence of \(\gamma\) carbonyl group of flavone, which may be assigned to C-4 position. The absorption peaks at \(\delta\) 56.01 and 56.6 ppm that may correspond to epoxy in form of angular C-O-C atoms at C-2'' and C-3'' positions. The peaks at \(\delta\) 61.00 ppm showed a sharp signal, assigned at C-4' due to presence of electron donating groups like (-OCH\(_3\)) in this structure. The \(^{13}\text{C}\)-NMR signals at \(\delta\) 162.6 and 104.6 were attributed to unsaturation of C-2 and C-3 in isolated flavones structure.

The \(^1\text{H}\)-NMR spectrums displayed sharp aromatic proton signals \(\delta\) H: 8.18 (1H, m, ArH-5), 7.47 (1H, m, ArH-6), 7.56 (1H, m, H-7), 7.55 (1H, d, H-8), suggested a flavone skeleton. The singlet proton signal also observed at \(\delta\) 6.54 which may be contributed due to presence of H-3 cyclic unsaturated ketone system. The singlet proton signal observed at \(\delta\) 3.31 ppm may be attributed to the presence of the group of methoxy at the position C-4. Two functional ether groups present in same structure were distinguished by \(^1\text{H}\)NMR spectra as methoxy and epoxy groups. The epoxy protons signal appeared at \(\delta\) 2.89 and 3.81 which were assigned to H-2'' and H-3'' positions which was also supported by IR stretching at 1175.40 cm\(^{-1}\).

GC-MS spectroscopy showed a molecular ion [M]\(^+\) peak at 368.13 m/z value corresponding \([\text{C}_{21}\text{H}_{20}\text{O}_6]\) which confirms the proposed molecular formula. The Chemical name of this flavones derivative is [3''-(3''-(hydroxymethyl) oxiran-2''-yl)-2'-methoxy-4''-(methoxymethyl)phenyl]-4H-chromen-4-one and were supported by the molecular weight 368.38 calculated by Rast’s procedure. It also showed a series of peaks at 137, 163, 252, 271, 268, 297, 337 and 368 amu corresponding to various fragments. Fragmentation ion peaks at m/z 337 correspond to the loss of -CH\(_2\)OH (M-31) group from the C-3'' position of the epoxy substituent of parent molecule. The next fragment ion peak at m/z297 (M-40) corresponds to the removal of epoxy ethane from
the C-5’ of the parent molecule. Moreover, the two signals exhibited at m/z 271 and 268, from which one should be attributed either due to the loss of -CH₂OCH₃ moiety from the 4’-carbon atom position or attribute the de-methoxylated fragment ion (M-31) from the 2’-carbon atom position peak. The fragment signals at m/z 252 appeared due to an equatorial cleavage of isolated flavones structure. Hence from the above discussion, it was concluded that the isolated molecule contains flavone moiety attached to substituted hydroxyl methyl oxirane at C-5’ position and methoxy methyl at the C-4’ position. The spectral data such as ¹³C-NMR, ¹H-NMR and Mass spectra were reported in the present study led us to the molecular formula of the compound as C₂₁H₂₀O₆, bearing the IUPAC nomenclature 2-[(3’-[3”-(hydroxymethyl)oxiran-2”-yl]-2’-methoxy-4’-(methoxymethyl)phenyl]-4H-chromen-4-one.

So, the present study justifies the folklore claims on *Avicennia alba* Blume for its antifertility activity. In addition, the plant is found to possess analgesic, antipyretic, muscle relaxant, antimicrobial and anti oxidant activity.